

Crystallization and Structural Determination of the Human Glucose Transporters GLUT1 and GLUT3

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Abstract

Overexpression, purification, and crystallization of eukaryotic membrane proteins represent a major challenge for structural biology. In recent years, we have solved the crystal structures of the human glucose transporters GLUT1 in the inward-open conformation at 3.17 Å resolution and GLUT3 in the outward-open and occluded conformations at 2.4 and 1.5 Å resolutions, respectively. Structural elucidation of these transporters in three distinct functional states reveal the molecular basis for the alternating access transport cycle of this prototypal solute carrier family. It established the molecular foundation for future dynamic and kinetic investigations of these GLUTs, and will likely facilitate structure-based ligand development. In this chapter, we present the detailed protocols of recombinant protein expression, purification, and crystallization of GLUT1 and GLUT3, which may help the pursuit of structural elucidation of other eukaryotic membrane proteins.

Key words Glucose transporters, Glut, GLUT1, GLUT3, Protein purification, Crystallization

1 Introduction

Structural biology aims to unveil the biological world at atomic scale. X-ray crystallography, electron microscopy (EM), and nuclear magnetic resonance (NMR) are the major experimental approaches for elucidating the three-dimensional structures of macromolecules. Among all the biological molecules and miniature machineries, integral membrane proteins, particularly eukaryotic membrane proteins, represent the most challenging targets for structural biology due to the technical difficulties associated with protein generation, purification, and crystallization. By October 4th 2016, in total 2052 structures of membrane proteins were reported, representing approximately 1.5 % of the total structure entries deposited to Protein Data Bank (PDB). Among these structures, only 647 are unique ones (<http://blanco.biomol.uci.edu/mpstruc/>).

The recent technological breakthrough of cryo-EM has drastically promoted structural elucidation of membrane proteins with a large molecular mass. However, for those proteins with a molecular weight below 150 kDa, the primary method for high-resolution structure resolution remains to be X-ray crystallography.

The SLC2A family glucose transporters, exemplified by GLUT1, 2, 3, and 4, have been the prototype in the investigation of solute transport. GLUT1–4 catalyze facilitative diffusion of glucose across biomembranes, responsible for the supply of glucose to brain and other organs [1]. Owing to their fundamental physiological significance, structures of GLUTs have been pursued for decades. In the past several years, we have solved four X-ray structures of human GLUT1 and GLUT3 at three distinct transport states, including GLUT1 in the inward-open conformation at 3.17 Å, and GLUT3 in the maltose-bound outward-open state at 2.6 Å and occluded conformations in the presence of glucose or maltose at 1.5 and 2.4 Å resolutions, respectively. With these structures, a morph of a nearly complete alternating access transport cycle can be generated.

To capture the structures of GLUT1 and GLUT3 at different functional states, we aimed to set up efficient strategies for (1) the overexpression of GLUTs in insect cells, (2) the purification with appropriate detergents, (3) crystallization, and (4) structure determination. We hereby also describe a proteoliposome-based counter-flow assay following a modified protocol [2] to qualitatively examine the transport activity of GLUTs. In retrospect, the successful crystallization and structural determination of GLUT1 and GLUT3 in different conformations may be attributed to the following elements:

1. Introduction of point mutations that remove glycosylation. We have introduced glycosylation-eliminated variants of GLUT1 (N45T) and GLUT3 (N43T).
2. Introduction of point mutation to GLUT1 that may lock the protein in the inward-open conformation. By literature search, we identified one single-point mutation GLUT1 (E329Q) that may lock the protein in an inward-open state. The mutation was originally identified as a disease-related variation in GLUT4.
3. The recombinant GLUT1 protein was purified and crystallized in the presence of the detergent β -nonyl-D-glucopyranoside, which may further stabilize an inward-open conformation of the transporter.
4. Crystallization of GLUT1 was carried out at 4 °C, which may lower the mobility of the transporter, facilitating crystallization.

2 Materials

1. Modified vectors from pFastBac1 (Thermo).
2. *E. coli* strains: DH5 α and DH10Bac.
3. Insect cell lines: Sf-9 (*Spodoptera frugiperda*), High Five (*Trichoplusia ni*).
4. Luria-Bertani (LB) medium: LB Broth, Miller.
5. LB agar plates (for plasmid generation): LB agar plates containing 100 $\mu\text{g}/\text{mL}$ ampicillin (Amresco).
6. LB agar plate (for bacmid generation): LB agar plates containing 100 $\mu\text{g}/\text{mL}$ x-gal, 40 $\mu\text{g}/\text{mL}$ IPTG, 50 $\mu\text{g}/\text{mL}$ kanamycin, 7 $\mu\text{g}/\text{mL}$ gentamicin, 10 $\mu\text{g}/\text{mL}$ tetracycline.
7. LB medium (for bacmid generation): LB medium with 50 $\mu\text{g}/\text{mL}$ kanamycin, 7 $\mu\text{g}/\text{mL}$ gentamicin, 10 $\mu\text{g}/\text{mL}$ tetracycline.
8. Ethanol solution: 70 % v/v Ethanol.
9. Antibiotic solution: 10 \times Penicillin-streptomycin solution.
10. HyCloneTM SFX-Insect cell culture medium (GE Healthcare).
11. Cellfectin II (Thermo).
12. SIM HF cell culture medium (Sino Biological Inc.).
13. FBS solution: 5 % v/v FBS (Thermo).
14. Lysis buffer 1: 25 mM Tris-HCl pH 8.0 and 150 mM NaCl.
15. Lysis buffer 2: Lysis buffer 1 containing protease inhibitors (0.8 μM aprotinin, 2 μM pepstatin, 5 $\mu\text{g}/\text{mL}$ leupeptin) and 2 % w/v n-dodecyl- β -D-maltoside (DDM).
16. Ni-NTA agarose.
17. Wash buffer: 25 mM MES pH 6.0, 150 mM NaCl, 30 mM imidazole, 5 % v/v glycerol, and 0.05 % w/v n-dodecyl- β -D-maltopyranoside (DDM) (Anatrace).
18. Elution buffer: 25 mM MES pH 6.0, 150 mM NaCl, 300 mM imidazole, 5 % v/v glycerol, and 0.05 % w/v DDM.
19. Centrifugal filters: 50 kDa Molecular cutoff.
20. Size-exclusion buffer: 25 mM MES pH 6.0, 150 mM NaCl, 5 % v/v glycerol, and 0.4 % w/v n-Nonyl- β -D-glucopyranoside (β -NG) (Anatrace).
21. Anti-His tag mouse polyclonal antibody, goat anti-mouse IgG, HRP-conjugated IgG (CWBio), and TMB substrate.
22. Phusion DNA Polymerase (Thermo).
23. T4 DNA ligase (NEB).
24. dNTP solution: 10 mM dNTP.

25. TIANgel Mini Purification Kit (TIANGEN).
26. DNA product Purification Kit.
27. Plasmid Purification Kit.
28. Reservoir solution (for hanging-drop vapor diffusion crystallization of GLUT1): 30 % w/v PEG400, 0.1 M MES pH 6.0, and 0.1 M MgCl_2 .
29. Mother liquor (for lipid cubic phase (LCP) crystallization of GLUT3): 28 % v/v PEG400, 0.1 M HEPES pH 6.8, and 50 mM ammonium citrate.
30. Precipitant solution (outward-occluded conformation): 38–40 % v/v PEG 400, 100 mM $(\text{HCOO})_2\text{Mg}$, 50 mM maltose, and 100 mM ADA pH 6.5.
31. Precipitant solution (outward-open conformation): 34 % v/v PEG 400, 400 mM $(\text{NH}_4)_2\text{HPO}_4$, 50 mM maltose, and 100 mM ADA pH 6.9.
32. MicroMesh (M3-L18SP-50; MiTeGen).
33. Radioactive glucose: 1 μCi D-[2- ^3H] glucose (specific radioactivity 21.5 Ci/mmol, 0.46 mM external D-[2- ^3H] glucose) (Perkin Elmer).
34. *E. coli* polar lipids extract (Avanti Polar Lipids Inc.).
35. Mixed liposome solution: 3:1 v/v chloroform and methanol.
36. KPM buffer: 50 mM Potassium phosphate pH 6.5, 2 mM MgSO_4 .
37. PC Membranes 0.4 μm (Satorius), GSTF membrane filter 0.22 μm (Merck-Millipore).
38. Bio-Beads SM2 (Bio-Rad).
39. Optiphase HISAFE 3 (PerkinElmer).
40. MicroBeta JET (PerkinElmer).
41. Glass sandwich plates (Shanghai FAsTAL BioTech).
42. Robot arm Gryphon (ARI).
43. SPEXTM 6770PLUS (SPEX SamplePrep).
44. SDS-polyacrylamide gel equipment.
45. Superdex-200 10/300 GL.
46. HiTrap Desalting column, 5 mL.
47. ÄKTA pure chromatography system (GE Healthcare).

3 Methods

The general steps required for the structure determination of GLUTs. The methods comprise (1) the establishment of Bac-to-Bac® Baculovirus Expression System for expression of GLUTs in

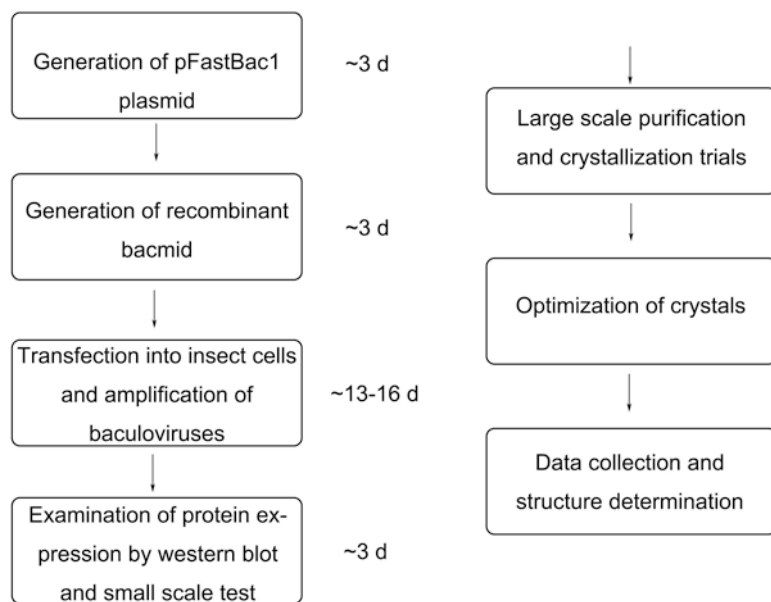


Fig. 1 General steps for structure determination of GLUTs. This flow chart presents the procedure for determining the structure of GLUT1/GLUT3. Each step will be described in the following parts. Also shown is the approximate time cost for major steps involved

insect cells, (2) protein expression and purification, (3) crystallization, and (4) data collection and structure determination (see Fig. 1). In this chapter, we only mention the case using insect cells; however GLUT1 was also purified from the overexpression system of yeast (*Saccharomyces cerevisiae* [3] and *Pichia pastoris* [4]) and human erythrocyte membrane [5].

3.1 Generating the Recombinant pFastBac Vector

For GLUT1, the full-length human GLUT1 cDNA is subcloned into the NdeI and XhoI sites of a modified pFastBac vector with a standard PCR-based strategy. A C-terminal 10× His-tag immediately follows the sequence. For GLUT3, the synthesized and codon-optimized cDNA of human GLUT3 (N43T) is subcloned into a modified pFastBac vector with an N-terminal 10× His-tag. The pFastbac vectors used in our study were modified to introduce the His-tag to GLUTs (see **Note 1** and Fig. 2).

1. The cDNAs of GLUTs are amplified using a standard PCR protocol. The PCR reaction mixture, 50 μ L in total, is composed of 1 μ L (200 ng) of DNA template, 50 pmol of each primer, 0.5 μ L of Phusion enzyme, 2 μ L dNTP, and 1 μ L DMSO in the phusion buffer.
2. The PCR products are purified with the TIANgel Mini Purification Kit.

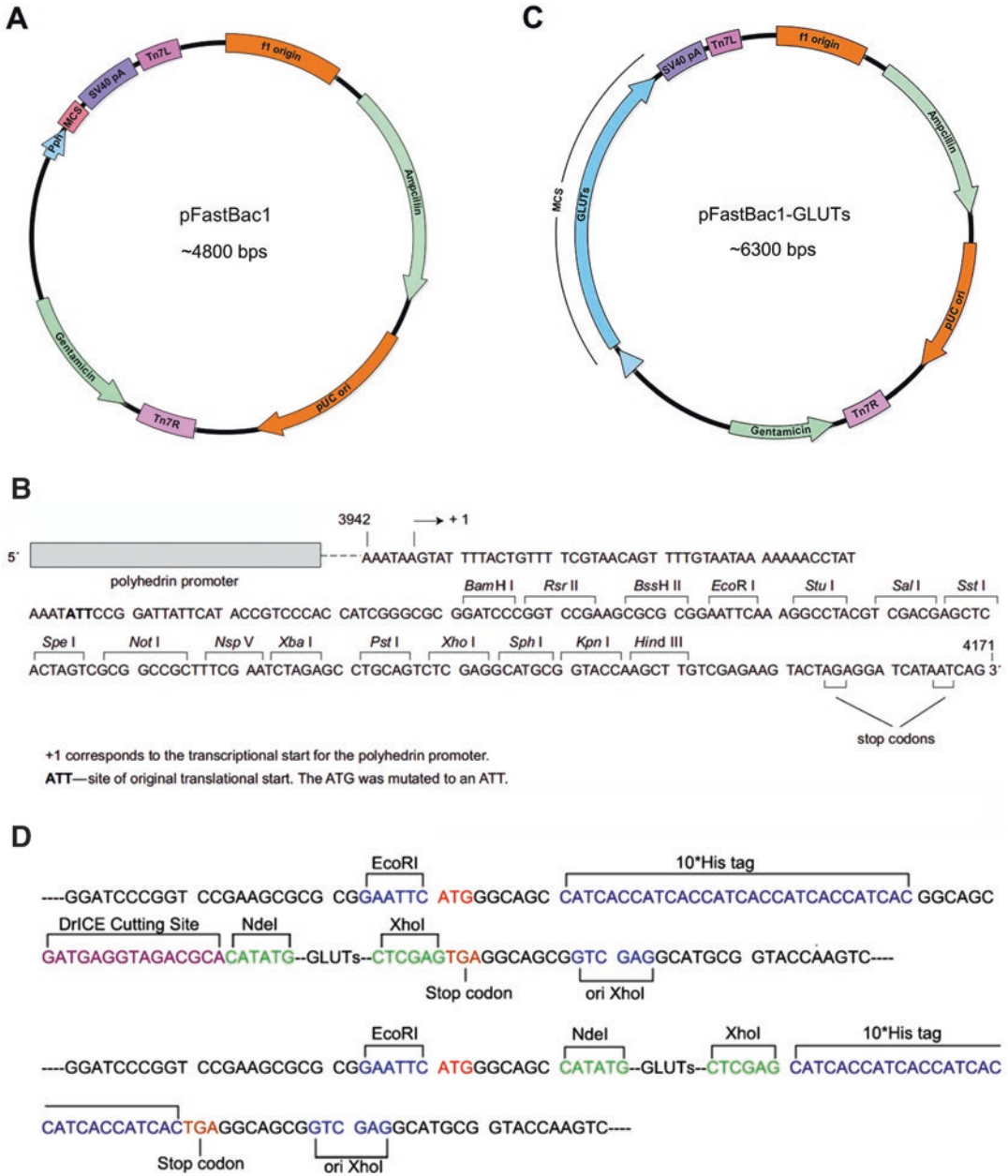


Fig. 2 Maps of plasmids used in the experiments. (a) Map of commercial pFastBac1 plasmid. (b) Sequence of original MCS in pFastBac1. (c) Map of recombinant plasmid (pFastBac1-GLUTs) with vector reconstructed. (d) Detailed reconstruction of the pFastBac1-GLUTs at MCS. The original RE cutting site, the constructed RE cutting site, and the 10× His tag and protease cutting site are shown in *light blue*, *green*, *dark blue*, and *purple*, respectively

3. The purified PCR products and vectors are digested by *Nde*I and *Xho*I for 1.5 h at 37 °C. The restricted digestion reaction, 60 µL in total, is composed of 52 µL PCR products or 2 µg vectors, 1 µL *Nde*I, and 1 µL *Xho*I in Cutsmart buffer.
4. The digested PCR products are purified with the DNA product purification kit. The vectors are purified with the TIANGel Mini Purification Kit.
5. The purified PCR products and vectors are ligated using T4 ligase. The ligation reaction, 10 µL in total, is composed of 1 µL T4 ligase, 1 µL digested vectors (20 ng), and 7 µL digested DNA (~100–200 ng) insert in ligation buffer.
6. Once the insert is integrated into the vector, the ligation reaction is transformed into DH5α *E. coli* and selected for ampicillin-resistant transformants on LB agar plates (amp⁺).
7. To identify the correct colony, positive transformants are analyzed by PCR and further checked with sequencing.
8. The colony is purified with the Plasmid Purification Kit to generate the pFastBac-GLUTs plasmids.
9. For mutagenesis of GLUT1 N45T/E329Q, and GLUT3 N43T, the quick-change methods were carried out with complementary primers comprising mutations following a modified protocol (*see Note 2*).

3.2 Generating the Recombinant Bacmids

1. For transformation, 50 µL DH10Bac competent cells are thawed on ice in advance.
2. 1 µL pFastBac-GLUT plasmids (about 400 ng) are added into the competent cells and mixed gently.
3. Incubate the transformed cells on ice for 30 min. Heat shock the cells at 42 °C for 90 s and then immediately chill on ice for 2–5 min.
4. Add 600 µL of LB medium without antibiotics and recover the cells at 37 °C at 220 rpm for 3 h.
5. Plate 25 µL, 50 µL, and 100 µL, respectively, of the cells on a LB agar plate (for bacmid generation). Incubate the plate for at least 48 h at 37 °C. To identify the correct colonies, single white bacterial colonies are analyzed by PCR with pUC/M13 forward and reverse primers (*see Note 3*).
6. Incubate the correct colonies in 3 mL LB medium (bacmid) at 37 °C overnight in a shaking incubator. Collect the bacterial cells by centrifugation and purify the recombinant bacmids with plasmid purification kit following the manufacturer's manual, but instead of using the column to purify the bacmid, precipitate the DNA with isopropanol. Do this by adding 640 µL isopropanol into 800 µL supernatant after centrifugation, and incubate the mixture in –20 °C for 60 min.

7. To collect the precipitated DNA, the solution is centrifuged at $13,000 \times g$ at 4 °C for 15 min.
8. Wash the pellet with 1 mL ethanol solution followed by 1 mL 100 % ethanol.
9. Dissolve the recombinant bacmids in 50 μ L sterile ddH₂O. Do not pipette or vortex the solution.
10. Run a 0.5 % w/v agarose gel to check the purified bacmid DNA (large amounts of plasmids can also be found) as well as the additional PCR analysis with the pUC/M13 primers (pUC/M13 forward: 5'-CCCAGTCACGACGTTGTAACACG-3'; pUC/M13 reverse: 5'-AGCGGATAACAATTTTCACACAGG-3') to confirm that the bacmids contain the target genes.

3.3 Producing Recombinant Baculovirus

Transfecting the recombinant bacmids into the Sf-9 cells produces recombinant baculovirus.

1. Sf-9 cells are cultured in Hyclone SFX insect cell culture medium. Add 1 mL Sf-9 cells ($\sim 1.5 \times 10^6$ cells/mL) and 1.5 mL medium in 60 mm tissue culture plates, allowing cells to attach for 10 min at 27 °C. Wash the attached cells once with 2 mL Hyclone SFX insect cell culture medium and add another 2 mL Hyclone SFX insect cell culture medium (*see Note 4*).
2. Dilute 10 μ L of recombinant bacmids into 100 μ L of Hyclone SFX insect cell culture medium, and 6 μ L of cellfectin II in 100 μ L of Hyclone SFX insect cell culture medium, respectively. Combine the diluted DNA and cellfectin II and mix gently. Incubate the mixture for 15–30 min at room temperature.
3. Add the mixture dropwise onto the cells and incubate for 4 h at 27 °C. Remove the mixture and add 2.5–3 mL of the Hyclone SFX insect cell culture medium with antibiotic solution and FBS solution. Incubate the cells at 27 °C for 72–96 h.
4. Collect the medium containing the virus. Centrifuge the medium at $800 \times g$ for 5 min to collect the supernatant and remove the precipitates. Store the clarified supernatant (P1 viral stock) in a fresh conical tube at 4 °C away from light.
5. To obtain higher titer, the baculoviral stock needs to be amplified from P1. Add 5–7 mL Sf-9 cells ($\sim 1.5 \times 10^6$ cells/mL) in 150 mm tissue culture plates with 35–40 mL fresh Hyclone SFX insect cell culture medium with antibiotic solution and add P1 baculoviral stock at the ratio of 1:20. Incubate the cells at 27 °C for 48–72 h in humidified incubator. Collect the medium containing the virus.

6. Centrifuge the medium at $800 \times g$ for 5 min in a sterile 50 mL conical tube to remove the precipitates.
7. Store the clarified supernatant (P2 viral stock) with additional 2–3 % serum in a fresh 50 mL conical tube at 4 °C, away from light (*see Note 5*).
8. The P3 or P4 viral stocks are generated from P2 or P3, respectively, and accomplished by repeating this protocol.

3.4 Expressing Recombinant GLUTs Proteins

Before large-scale expression of GLUTs, it is necessary to detect the expression by Western blotting for a preliminary analysis. Other screening systems, such as the fluorescence-detection size-exclusion chromatography for GFP-tagged proteins, are also available [6]. Here, we only mention the protocol for Western blotting analysis and the following large-scale expression.

1. After transfection with bacmid, the Sf-9 cells generate virus and meanwhile start to express the GLUT proteins.
2. Collect the Sf-9 cells in the step of generating P3 or P4 viral stock and lyse the cells with $2\times$ SDS loading buffer.
3. Analyze the cell lysates by SDS-PAGE and Western blotting. Use the anti-His antibody to detect the GLUT protein which carries an N- or a C-terminal $10\times$ His-tag.
4. For large-scale expression of GLUT1 (N45T/E329Q) and GLUT3 (N43T) we recommend to grow cells in suspension using the High Five and Sf-9, respectively.
5. Maintain the cells in a 27 °C shaker. Determine the viable cell count from a 3-day-old suspension culture and dilute the cell suspension to 0.8×10^6 cells/mL in the SIM HF medium for High Five cells or SFX medium for Sf-9 cells in a 2 L flask.
6. Shake the cells until their density reaches 1.5×10^6 cells/mL.
7. Add 15–25 mL baculoviral stock (P3 or P4) to 800 mL culture to infect the cells and incubate for 48 or 72 h in a 27 °C shaker (*see Note 6*).
8. Harvest the cells by centrifugation at $3,000 \times g$ for 10 min at 4 °C and remove the media. The cell pellets are ready for protein purification.

3.5 Protein Purification

Purification of GLUT1 and GLUT3 (*see Fig. 3*) follows the same approach, except with minor modifications (*see Note 7* and *Note 8*).

1. Forty-eight hours after viral infection, the High Five cells of 4 L cultures are collected.
2. Resuspend the cells in 100 mL lysis buffer. The collected cells can either be immediately used or frozen in liquid nitrogen and then stored at -80 °C.

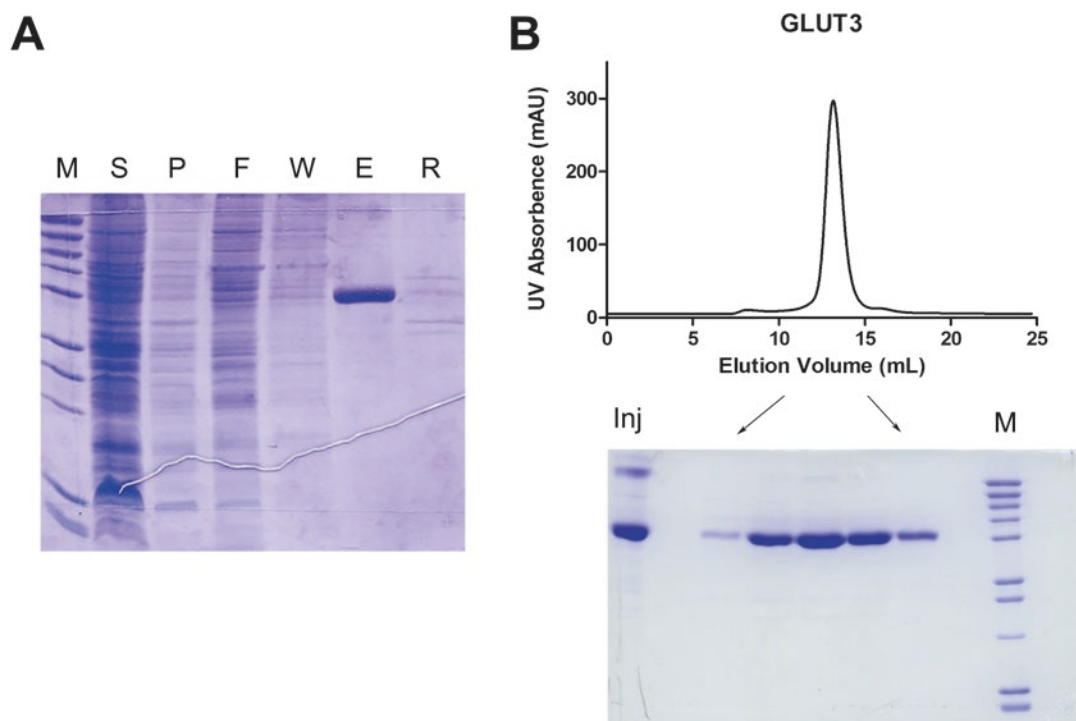


Fig. 3 A typical example for purification of GLUT3 by Ni-NTA and size-exclusion chromatography. **(a)** Samples from the Ni-NTA purification were applied for SDS-PAGE followed by Coomassie blue staining. *M* protein marker, *S* supernatant after ultracentrifugation, *P* pellet after ultracentrifugation, *F* flow through, *W* wash, *E* elution, *R* resin of Ni-NTA. **(b)** Result of size-exclusion chromatography, the sample before injection (INJ) and peak fractions were applied for SDS-PAGE analysis

3. Disrupt the cells using Dounce homogenizer for 80 cycles on ice. After centrifuging the total extract at $5,000 \times g$ for 10 min followed with ultracentrifugation of the supernatant at $150,000 \times g$ for 1 h at 4°C , the membrane fraction is solubilized in lysis buffer II at 4°C for 2 h.
4. Ultracentrifuge the membrane fraction at $150,000 \times g$ for 30 min, and then incubate the detergent-soluble fraction with nickel affinity resin (Ni-NTA) at 4°C for 30 min.
5. Rinse the resin with the wash buffer three times.
6. Elute the protein from the affinity resin with the elution buffer (*see Note 7*).
7. Concentrate the protein to about 10 mg/mL using a 50 kDa centrifugal filter.
8. Further purification of GLUT1 protein is carried out in the size-exclusion buffer by gel filtration. Collect the peak fractions and flash freeze the protein in liquid nitrogen and then store at -80°C for following crystallization trials and biochemical assay (*see Note 8*).

3.6 Protein Crystallization

GLUT1 and GLUT3 were crystallized in different manners. We provide here the individual protocols that led to the successful crystallization of GLUT1 and GLUT3, respectively.

3.6.1 Crystallization of GLUT1 (N45T/E329Q)

For crystallization of GLUT1, the hanging-drop vapor diffusion method is carried out.

1. Pipette 0.3 mL crystallization buffer into the reservoir of the 24-well plate (such as the VDX plate).
2. Pipette 0.8 μ L of the GLUT1 solution into the center of a siliconized 22 mm square cover slide.
3. Pipette 0.8 μ L of buffer from the reservoir into the drop on the cover slide.
4. Invert the cover slide so that the drop will be hanged from cover slide and position the cover slide onto the bead of grease on the reservoir. Press the slide down gently and ensure a complete seal.
5. The initial crystallization screening of GLUT1 is set up using commercial screening kits, including MemGold I/II, MemStart, MemSys, and MemPlus (Molecular Dimensions).
6. Most of the extensive crystallization trials failed to yield crystals for GLUT1 purified in various detergents. The crystals did however appear in the reservoir solution (GLUT1) at 4 °C after 2 days and reached full size in 5–7 days. Collect the crystals using Mounted Cryoloop with 20 μ m diameter nylon (Hampton Research) and flash freeze the crystals in liquid nitrogen immediately.

3.6.2 Crystallization of GLUT3 (N43T)

GLUT3 (N43T) in complex with D-glucose or maltose was crystallized with the lipidic cubic phase approach. The following protocol explains this approach.

1. The protein is concentrated to 30–40 mg/mL, and then mixed with monoolein in ratio 1:1.5 w/w proteins:lipid ratio using a syringe lipid mixer.
2. For crystallization of GLUT3 (N43T) in complex with D-glucose, the robot arm Gryphon (ARI) was used to mix the 40 nL meso phase with 900 nL crystallization buffer for each condition on glass sandwich plates.
3. The crystals appeared within 1 week with a typical size of 70 μ m \times 50 μ m \times 10 μ m.
4. These crystals diffracted X-rays to approximately 2.5 Å at SSRF beamline BL17U. Mother liquor is used to optimize the crystals, which in our hands gives rise to crystals with an approximate size of 140 μ m \times 100 μ m \times 20 μ m.

5. For crystallization of GLUT3 (N43T) in complex with maltose, 30–45 nL meso phase was overlaid with 800 nL of precipitant solution. In our hands, crystals appeared overnight and grew to a maximum size of about $20\text{ }\mu\text{m} \times 20\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ at $20\text{ }^{\circ}\text{C}$ within 1 week.
6. Collect the crystals using MicroMesh and flash freeze the crystals in liquid nitrogen immediately.

3.7 Data Collection and Structure Determination

All data sets of GLUT1 and GLUT3 were collected at BL17U at SSRF and the microfocus beamline BL32XU at SPring-8, respectively (*see* **Note 9**).

3.8 Preparation of Liposomes and Proteoliposomes

Liposomes can be prepared according to general protocols.

1. Dissolve 20 mg *E. coli* polar lipid extract into chloroform to a final concentration of 50 mg/mL in a glass vial. Evaporate the solvent and make sure that the lipids are distributed over the wall and bottom evenly using a stream of ultrapure nitrogen gas to prevent the lipids from oxidation.
2. Resuspend the lipids with the KPM buffer plus 50 mM glucose and vortex to make an emulsion at 20 mg/mL.
3. Freeze the liposomes in liquid nitrogen and thaw again. Repeat this for ten cycles. Then extrude the liposomes through a membrane filter.
4. For the counter-flow assay of GLUTs, proteoliposomes can be prepared using the 20 mg/mL pre-extruded liposome suspension.
5. Incubate the liposomes with 1 % w/v n-octyl- β -D-glucopyranoside (β -OG) for 60 min at $4\text{ }^{\circ}\text{C}$.
6. Add the purified GLUT1 (N45T) or GLUT3 (N43T) (10 μg protein per mg lipid) and incubate for an additional 60 min at $4\text{ }^{\circ}\text{C}$.
7. To remove β -OG, the sample is incubated with 240 mg/mL Bio-Beads SM2 overnight and then with 120 mg/mL Bio-Beads for an additional 2 h. Removal of detergent reconstitutes the protein into the liposomes to produce proteoliposomes.
8. Freeze the proteoliposomes in liquid nitrogen and thaw. Repeat for five cycles, and extrude the sample through a membrane filter.
9. Collect the proteoliposomes by ultracentrifugation at $100,000 \times g$ for 1 h, and wash with ice-cold KPM buffer to remove the excessive glucose. Resuspend the proteoliposomes with KPM buffer to a final concentration of 100 mg/mL (phospholipids).

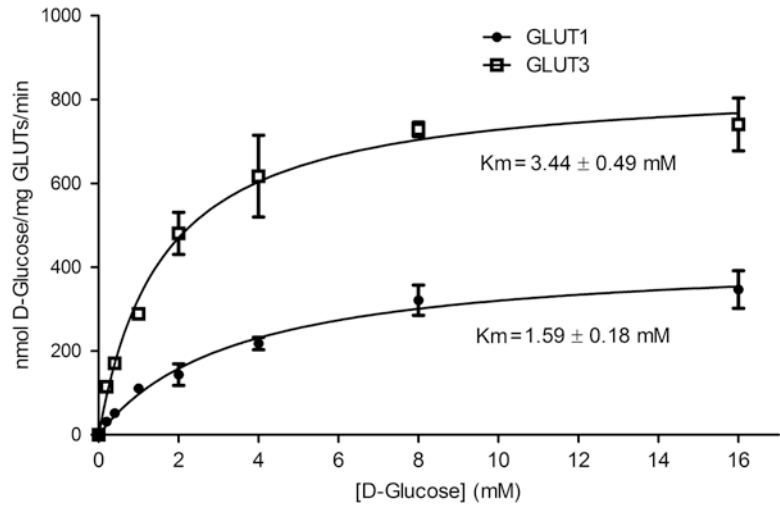


Fig. 4 Detected kinetic parameters of GLUT1 and GLUT3 by in vitro proteoliposome-based assay. K_m and V_{max} values of these GLUTs were detected with the described protocol. Different concentrations of unlabeled glucose (“cold”) were added in the reaction buffer. Samples were taken each 10 s to monitor the V_0 of GLUTs. Data points were done in triplicates. Error bars represent standard deviations

3.9 Counter-Flow Assay

1. Add 2 μ L prepared proteoliposomes of GLUT1 or GLUT3 into 100 μ L KPM buffer plus radioactive glucose, 1 μ Ci D-[2- 3 H] glucose.
2. After incubation, stop the uptake of radiolabeled substrates by filtering the sample through 0.22 μ m Millipore filters.
3. Wash the filter membranes with 2 mL ice-cold KPM buffer immediately and solubilize the membranes with 0.5 mL Optiphas HISAFF 3. The solution is then used for liquid scintillation counting with MicroBeta JET.
4. The counter-flow assays are performed at 25 $^{\circ}$ C and repeated at least three times.
5. For determination of V_{max} and K_m , the initial velocities are measured at 10-s time intervals with a concentration gradient of D-glucose, which consists of a mixture of isotope-labeled and non-labeled (“cold”) D-glucose, in the outside buffer (see Fig. 4).
6. The data are then fitted to the Michaelis–Menten equation using GraphPad Prism 5.0.

4 Notes

1. It is more convenient to adjust the restriction enzyme (RE) cutting site and ideal tags in commercial vectors to unify the cloning system. Thus, we introduced the *Nde*I and *Xho*I site

to MCS of the pFastBac vector (digested with *EcoRI* and *Sall*), mediated by *EcoRI* and *Sall* (block original *XhoI*). For N-tag vector, 10× His-tag was added just before the *NdeI* cutting site with linker and a protease-cutting site (GS-10× His-SGDEVDA). For C-tag, 10× His-tag was added after *XhoI* cutting site followed with a stop codon. The recombinant plasmid is shown in Figure for a direct overview.

2. Complementary oligonucleotides containing the desired mutation are synthesized, flanked by 15 nt unmodified nucleotide sequence. 200–300 ng modified pFastBac-GLUT1 or GLUT3 is used as a template. Run the 50 µL PCR reaction for 30 cycles and use a 4-min extension. Generally, a higher annealing temperature (like 58 °C) gives a better result. Purify the PCR product using a kit. Add 1 µL of the DpnI restriction enzyme to 50 µL purified product with Cutsmart buffer, mix gently, and then incubate the reaction at 37 °C for 3–4 h to digest the methylated parental plasmids. The DpnI-treated DNA (10 µL/reaction) is then directly used for transformation into separate aliquots of the DH5α *E. coli* competent cells.
3. It is necessary to double check the recombinant bacmid generated in the DH10Bac *E. coli* using PCR analysis. It happens that white clones are false positive. The correct size of the PCR product should be ~2300 bp plus the size of the GLUT1 or GLUT3 for the bacmid transposed with our modified pFastBac. Moreover, the pair of primers for the target insert is not recommended for PCR analysis for the DH10Bac cell containing the recombinant plasmid pFastBac-GLUT1 or -GLUT3.
4. We recommend using Sf-9 cells for generating virus for the expression of GLUTs. Other cells may have less efficient transfection based on our experience. However, once the baculovirus stock is generated, Sf-9, High Five™, or Mimic™ Sf-9 cells can be used for expression trials.
5. The viral stocks are stored in aliquots at –80 °C for later re-amplification. Repeated freeze and thaw of the viral stock will result in dramatically decreased virus titer. Routinely used viral stocks should be stored at 4 °C, away from light.
6. The optimal time of protein expression is between 48 and 72 h. The cell morphology and density should be monitored to confirm the progress of infection.
7. The detergent used for GLUT3 is 0.05 % w/v DDM in the wash and the eluting buffer is replaced with 0.06 % w/v 6-cyclohexyl-1-hexyl-β-D-maltoside (CYMAL-6).
8. For crystallizing the GLUT3 in complex with D-glucose or maltose, 50 mM D-glucose or maltose is added throughout

the purification procedure. Concentrated GLUT3 protein is applied to HiTrap Desalting 5 mL in the 25 mM MES pH 6.0, 150 mM NaCl, 0.06 % w/v CYMAL-6 plus 50 mM D-glucose, or maltose.

9. Extended information about the data collection and structure determination can be found in refs [7, 8].

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