ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 1, pp. 75-80. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 1, pp. 95-102.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-163, November 9, 2008.

High Human GLUT1, GLUT2, and GLUT3 Expression in *Schizosaccharomyces pombe*

Yuxin Yang[1], Zongli Hu[1], Zhizhao Liu[1], Yi Wang[1], Xuqing Chen[1,2], and Guoping Chen[1]*

[1]*College of Bioengineering, Chongqing University, Shapingba, Chongqing 400044, China; fax: 0086-23-65112674; E-mail: chenguoping@cqu.edu.cn*
[2]*Beijing Research Center of Agro-Biotechnology, Beijing 100089, China*

Received May 7, 2008
Revision received May 28, 2008

Abstract—In this study, three subfamily members of the human 12-transmembrane-domain cell-surface receptors GLUT1, GLUT2, and GLUT3 were heterologously expressed in the fission yeast *Schizosaccharomyces pombe* utilizing GST–GLUT fusion proteins. These fusion proteins were driven by the full-length nmt1 promoter (Pnmt1) derived from *S. pombe*. The transcription levels of the GST–GLUT fusion proteins were very high upon induction by removing thiamine from the media. One-step purification of the recombinant fusion proteins was achieved by GST-affinity chromatography. Approximately 300 μg of highly purified fusion protein were obtained from 3 g of wet cell paste (1 liter of cell culture), indicating that human membrane proteins can be efficiently expressed and purified in the fission yeast. With its available extensive genetic information and ease of genetic manipulation, the fission yeast is potentially a highly efficient host to express eukaryotic membrane proteins.

DOI: 10.1134/S0006297909010118

Key words: GLUT, *Schizosaccharomyces pombe*, membrane protein expression, GST-affinity chromatography

The recombinant expression and subsequent purification of integral membrane proteins is considered a major challenge, especially for eukaryotic membrane proteins. For instance, overexpression in both heterologous and endogenous hosts is often toxic and can result in the production of inactive proteins or insoluble aggregates [1]. Proper insertion into the host membrane is also rate-limiting and remains difficult to predict or regulate [2]. The functional and structural analysis of membrane proteins has been hampered by the lack of sufficient quantities of active proteins. Consequently, many investigators have attempted to deal with these difficulties by focusing on expression, purification, and crystallization strategies [3, 4].

The human GLUT1, GLUT2, and GLUT3 proteins (facilitated glucose transporter, hereafter referred to as GLUTs) belong to a large superfamily of 12-transmembrane segment transporters. These transport proteins have unique tissue distributions and biochemical properties that underlie specific physiological functions [5, 6], and they are involved in the transport of a variety of hexoses and other carbon compounds [7]. GLUT1 is expressed at the highest levels in the endothelium of barrier tissues, such as blood vessels and the blood–brain barrier. GLUT2 is a low-affinity glucose transport protein and is expressed in hepatocytes, pancreatic β-cells, and the basolateral membranes of intestinal and renal epithelial cells. GLUT2 acts as a high-capacity transport system, which allows the uninhibited (non-rate-limiting) flux of glucose into or out of these cell types. GLUT3 is a low K_m isoform that is responsible for glucose uptake in neurons [6, 8].

GLUTs function in the cellular uptake of glucose and are key glucose metabolism regulators. Therefore, studies of GLUT structure and function, the regulation and inhibition of their activity, their roles as drug targets, and their antibody production are important for understanding the pathogenesis of disease and exploring new treatment strategies. Such studies are highly dependent on efficient *in vitro* GLUT expression. Unfortunately, like with most membrane proteins, the expression, separation, and purification of GLUTs are technically challeng-

Abbreviations: DAB, 3,3′-diaminobenzidine; EMM, Edinburgh minimal media; GLUTs, facilitated glucose transporters; GST, glutathione S-transferase; PVDF, polyvinylidene difluoride; YES, yeast extract plus supplements medium.

* To whom correspondence should be addressed.

ing, and thus far, there has been no report of a system for high *in vitro* expression of GLUTs.

We have selected an efficient expression system that maintains the biochemical properties of heterologous proteins and carries out post-translation modifications equivalent to their native counterparts. This expression system utilizes the fission yeast *Schizosaccharomyces pombe* to efficiently overexpress human proteins [9-11]. Here we report the successful expression of the human GLUT1, GLUT2, and GLUT3 proteins in *S. pombe*.

MATERIALS AND METHODS

The human brain and liver cDNAs, Taq DNA polymerase, PrimeSTAR^{TM} HS DNA polymerase, T4 DNA ligase, CIAP, restriction enzyme *Bam*HI, the pMD18-T vector kit, and JM109 competent cells of *Escherichia coli* were obtained from Takara (Japan). Triton X-100 and IGEPAL detergents were purchased from Sigma (USA). The GSTrap FF column was obtained from GE (USA). All culture powders (YPD, YES, and EMM), *S. pombe* (SP-Q01), and the pESP-2 plasmid were acquired from Stratagene (USA).

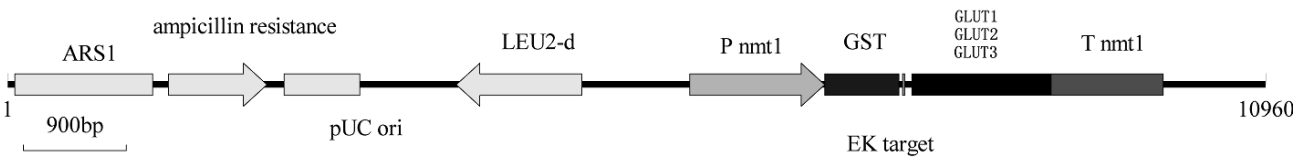
Construction of pESP-GLUT plasmids. The pESP-GLUT expression vectors were constructed from the plasmid pESP2 (Scheme). Three pairs of primers that contained *Bam*HI sites (underlined) on both the 5′ and 3′ ends were designed. Each 3′ primer sequence included a stop codon region (bold): GLUT1 (5′-GCGCGGATCCATGGAGCCCAGCAGCAAGAAGC-3′, 5′-GCGCGGATCC**TCA**CACTTGGGAATCAGCCCCC-3′); GLUT2 (5′-GCGCGGATCCATGACAGAAGATAAGGTCACTG-3′, 5′-GCGCGGATCC**TTA**CACAGTCTCTGTAGCTCCT-3′); GLUT3 (5′-GCGCGGATCCATGGGGACACAGAAGGTCACCC-3′, 5′-GCGCGGATCC**TTA**GACATTGGTGGTGGTCTCC-3′).

The GLUT1, GLUT2, and GLUT3 full gene fragments were obtained from cDNA by PrimerStar PCR. The reagents for a 50 μl PCR reaction were 32.5 μl of deionized water, 10 μl of 5× PS buffer, 4 μl of 10 mM dNTPs, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 0.5 μl of PrimerStar DNA polymerase (5 U/μl), and 1 μl of cDNA template. The thermal cycling parameters were 35 cycles of 98°C for 30 sec and 68°C for 1 min. These fragments were digested with *Bam*HI, gel purified, inserted into electroeluted *Bam*HI digested pESP-2, and transformed into JM109 *E. coli*. The resultant constructs encoded the respective full-length GLUTs fused in-frame (at the amino terminus) to a 222-amino acid residue of GST (glutathione S-transferase). These constructs were verified by DNA sequencing.

Transformation of *S. pombe* (SP-Q01). The method to prepare and transform competent SP-Q01 *S. pombe* cells was as described in the instruction manual of the ESP Yeast Protein Expression and Purification System and ESP Yeast Protein Expression Vectors (Stratagene).

Verification of transformation. Edinburgh minimal media (EMM)-thiamine agar plate selection: *leu1-32* was used as a selective marker to confirm efficient transformation in plasmid pESP-2. Transformants were identified by their pESP-GLUT vector-dependent ability to grow on a plate containing EMM supplemented with 25 mM thiamine. Colonies appearing on the EMM-thiamine agar plates within 4-6 days were verified by PCR for the presence of the GLUT inserts. The reagents used in a 25 μl PCR reaction were as follows: 17.5 μl of deionized water, 2.5 μl of 10× PCR buffer (Mg^{2+}-free), 1.5 μl of 2.5 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, 1 μl of 10 μM forward primer 5′-GTA CTT GAA ATC CAG CAA GTA TAT AGC-3′, 1 μl of 10 μM reverse primer 5′-CAA AAT CGT AAT ATG CAG CTT GAA TGG GCT TCC-3′ (this pair of primers was designed according to the pESP vectors near the *Bam*HI site), 0.25 μl of rTaq DNA polymerase (5 U/μl), and 1 μl of a dilution of a transformed colony in 10 μl of distilled water. The thermal cycling parameters were: 1 cycle of 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1.5 min, followed by a final 10 min at 72°C. The PCR reactions were analyzed by electrophoresis in a 1% agarose gel with 0.5 mg/liter of ethidium bromine. Transformants with correct-sized bands were selected.

Culture and expression. Five milliliters of yeast extract plus supplements medium (YES) with 5 μM of filter-sterilized thiamine was inoculated with a small patch of the transformed yeast cells. The yeast cell culture was incubated overnight in a 28°C incubator with shaking at 250 rpm. A 200-ml volume of YES medium with 5 μM of filter-sterilized thiamine was then inoculated with 1 ml of the overnight yeast culture and incubated at 28°C with shaking at 250 rpm until an OD_{600} value of 0.8-1.0 was obtained. Cells were harvested by centrifugation at 1000*g* for 5 min at room temperature, after which the super-



Sketch map of pESP-GLUT1, pESP-GLUT2, and pESP-GLUT3 expression plasmids. EK target, site for enterokinase

Scheme

natant was discarded. The cell pellet was washed at least twice by resuspension in 200 ml of sterile water, followed by centrifugation at 1000*g* for 5 min at room temperature and disposal of the supernatant. Expression was induced by resuspending the final yeast cell pellet in 200 ml of EMM broth. The yeast cell culture was subsequently incubated at 30°C with shaking at 200 rpm for 18-20 h.

Cell lysis and protein extraction. Yeast cells were harvested by centrifugation at 1000*g* for 5 min at room temperature, and cell pellets were transferred into a mortar. The pellets were rapidly frozen in liquid nitrogen, 0.5 mg of acid-washed glass beads was added, and the pellets were quickly and thoroughly ground. Extraction buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 300 mM NaCl, 0.5% IGEPAL, 100 mM NaF, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml aprotinin, 1 μM pepstatin A, 100 μM leupeptin, 1 μg/ml chymostatin in 800 μl) was added to the cells, and the extract was transferred to a fresh 2-ml microcentrifuge tube. Each cell extract was clarified by centrifugation at 13,000 rpm for 5 min at 4°C, after which each supernatant was transferred to a fresh 1.5-ml microcentrifuge tube.

Fusion GST–GLUT protein purification. Glutathione-Sepharose 4B MicroSpin columns (Amersham Biosciences, USA) were used to purify the GST–GLUT fusion proteins. The purification procedure was as described in the GST Spin-Trap Purification Module Product booklet, with one exception: the yeast cell lysate supernatant was mixed with Glutathione-Sepharose 4B for 60 min at 4°C. For the elution of the GST–GLUT fusion proteins, 100-200 μl of glutathione elution buffer were added to each column and incubated for 30 min at 4°C, twice. The eluate of the GST–GLUT fusion proteins was precipitated by adding an equal volume of methanol and incubating at –20°C for 30 min.

Calculation of protein concentrations and yield. Purified protein concentrations were determined as follows: measurements of UV absorbance at 280 nm (A_{280}) were carried out for the proteins in elution buffer, with the appropriate buffer blanks subtracted from the absorbance of the sample. Each sample was diluted 1 : 10. Extinction coefficients used to determine the protein concentrations were 95,855, 107,315, and 84,520 $M^{-1}\cdot cm^{-1}$ for GST–GLUT1, GST–GLUT2, and GST–GLUT3, respectively, as determined from protein sequences [12].

SDS-PAGE and Western blot analysis. SDS gel electrophoresis was performed in 12.5% acrylamide separating and 5% stacking gels containing 0.1% SDS to analyze the expression of the GST–GLUT fusion proteins. Gels were stained with Coomassie Brilliant blue. These proteins were not boiled before electrophoresis because certain membrane proteins aggregate irreversibly when heated, regardless of the presence of SDS in the sample buffer [3]. We observed the same phenomenon; when samples were boiled *prior to* loading, membrane protein expression could not be detected.

The samples were run on another 12.5% SDS-polyacrylamide gel and transferred to a PVDF (polyvinylidene difluoride) membrane through the electrophoretic transfer method. To block nonspecific binding sites, the PVDF membrane was immersed in a 1% BSA blocking reagent in PBST buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1% Tween) for 1 h on a platform shaker at room temperature. After rinsing the membrane in two changes of PBST, it was incubated in diluted anti-GST–HRP conjugate (1 : 5000) for 1 h at room temperature on the platform shaker. Subsequently, the membrane was washed in three changes of PBST at room temperature with gentle shaking, followed by the use of diaminobenzidine–hydrogen peroxide (DAB–H_2O_2) to visualize the signals.

In a separate immunoblot analysis, special anti-GLUT1, anti-GLUT2, and anti-GLUT3 antibodies were used at 1 : 100 dilution (these antibodies were gifts from Dr. W. G. Gwyn of University of Glasgow, UK). The visualization of bands achieved by incubation with anti-rabbit horseradish peroxidase (HRP) conjugate, followed by development using DAB–H_2O_2.

RESULTS

Construction and verification of pESP-GLUT plasmids. The pESP-GLUT expression vectors (Scheme) were constructed from the plasmid pESP2. Human GLUT1, GLUT2, or GLUT3 cDNA was inserted into this vector downstream of the nmt1 promoter. The nmt1 promoter is tightly repressed when fission yeast is cultured in media with thiamine (vitamin B1) over a concentration of 0.5 μM and is highly activated upon deprivation of thiamine in the media [13]. The SP-Q01 fission yeast strain selected for our protein expression and purification system had a *leu1-32* genotype. The *LEU2-d* gene from *Saccharomyces cerevisiae*, which has lacks its promoter, served as a selectable marker in the pESP-GLUT plasmids for the efficient transformation and maintenance of a high copy number of the expression constructs in the yeast cells [14]. In addition, the specificity of our PCR results showed that the pESP-GLUT plasmids were maintained in the selected transformants (Fig. 1).

Expression and purification of the GST–GLUT fusion proteins. For induction, the expression strains were grown to mid-log phase (A_{600} = 1.0) in YES media, which contained enough thiamine to repress the promoter. Expression of the GST fusion proteins was induced by harvesting cells and growing them in EMM media for 18-20 h. Cells were then lysed by grinding with glass beads, and the cleared lysate was used to purify the fusion proteins. One-step purification of the recombinant fusion proteins was achieved by GST-affinity chromatography. The purified fusion proteins were analyzed by SDS-PAGE. The four bands visualized were GST (synthesized in control cells

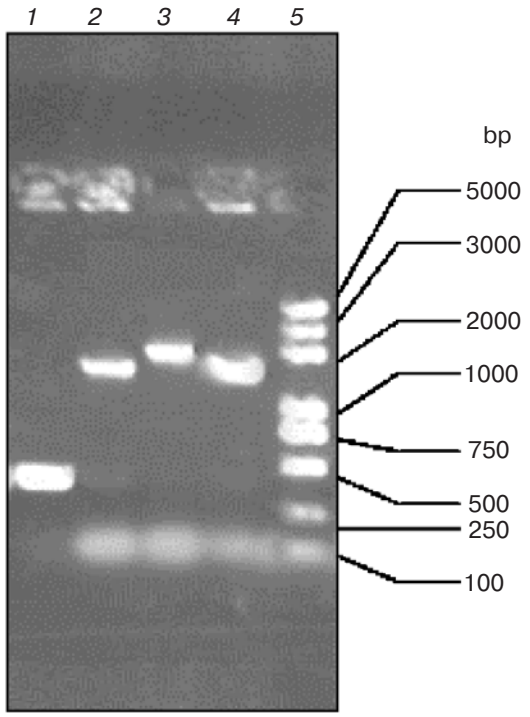


Fig. 1. PCR verification of the yeast transformants. The *GLUT1*, *GLUT2*, or *GLUT3* gene was inserted at the *Bam*HI site of pESP-2 vector and transformed into *S. pombe*. Colonies appearing on the EMM-thiamine agar plates within 4-6 days were verified by PCR. The primers were designed near the *Bam*HI site, and the fragment in the parental pESP-2 vector is approximately 280 bp in size. Lanes: *1*) control (synthesized in yeast cells carrying the parental pESP-2 vector); *2*) *GST–GLUT1*; *3*) *GST–GLUT2*; *4*) *GST–GLUT3*; *5*) marker.

carrying the parental pESP-2 vector), GST–GLUT1, GST–GLUT2, and GST–GLUT3 (Fig. 2).

Many membrane proteins migrate faster in SDS-PAGE than would be predicted from their molecular weights [15]. Here we also observed that the sizes of our expressed membrane proteins in electrophoresis were smaller than their actual molecular weights. The GST–GLUT1, GST–GLUT2, and GST–GLUT3 fusion proteins have predicted molecular masses of 80.4, 83.8, and 80.2 kD, respectively. However, electrophoretic estimates of the molecular masses of the proteins were 66 kD, with GST–GLUT2 being slightly larger than GST–GLUT1 and GST–GLUT3.

Western blot analysis of the fusion proteins. Due to the presence of the GST tail in the fusion proteins, immunoblot analysis was performed on the samples using an anti-GST–HRP conjugate to validate their identities (Fig. 3). In addition, an independent immunoblot analysis of each GLUT with its corresponding antibody also confirmed the identity of the expressed proteins. Bands were observed only when the corresponding GLUT antibody was incubated with the membrane containing GST (as control) and the three fusion proteins (Fig. 4).

We estimated that about 300 μg of highly purified GST–GLUT1 and GST–GLUT3 fusion proteins and 100 μg of GST–GLUT2 fusion protein were obtained from every 3 g of wet cell paste by GST-affinity resin.

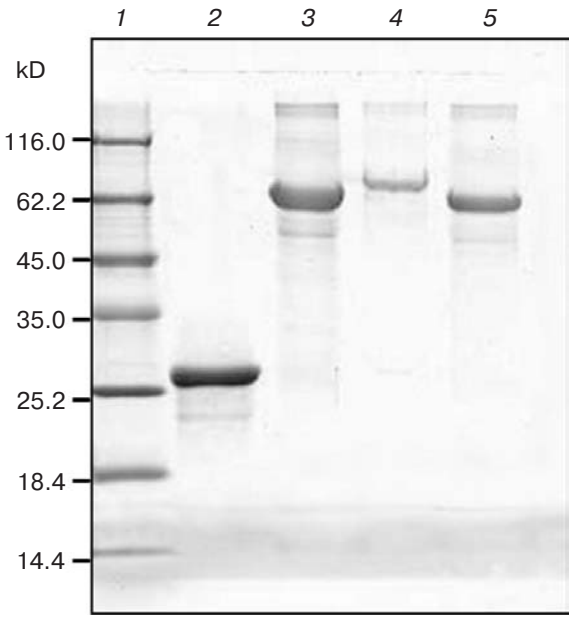


Fig. 2. Expression and purification of GST–GLUT fusion proteins. This figure shows a Coomassie blue-stained Tris-glycine SDS gel, with samples derived from yeast cells that were grown under induced conditions, after one-step purification by GST-affinity chromatography. Lanes: *1*) marker; *2*) GST (as a control, synthesized in control yeast cells); *3*) GST–GLUT1; *4*) GST–GLUT2; *5*) GST–GLUT3.

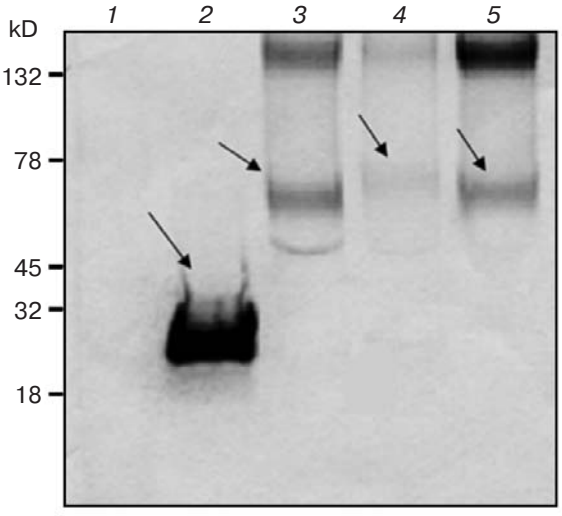


Fig. 3. Immunoblot analysis of the fusion proteins with a GST antibody. The SDS gel containing the purified GST–GLUT fusion proteins was transferred to a nitrocellulose membrane and subjected to Western analysis using the GST monoclonal antibody as the primary antibody. Lanes: *1*) marker; *2*) GST; *3*) GST–GLUT1; *4*) GST–GLUT2; *5*) GST–GLUT3.

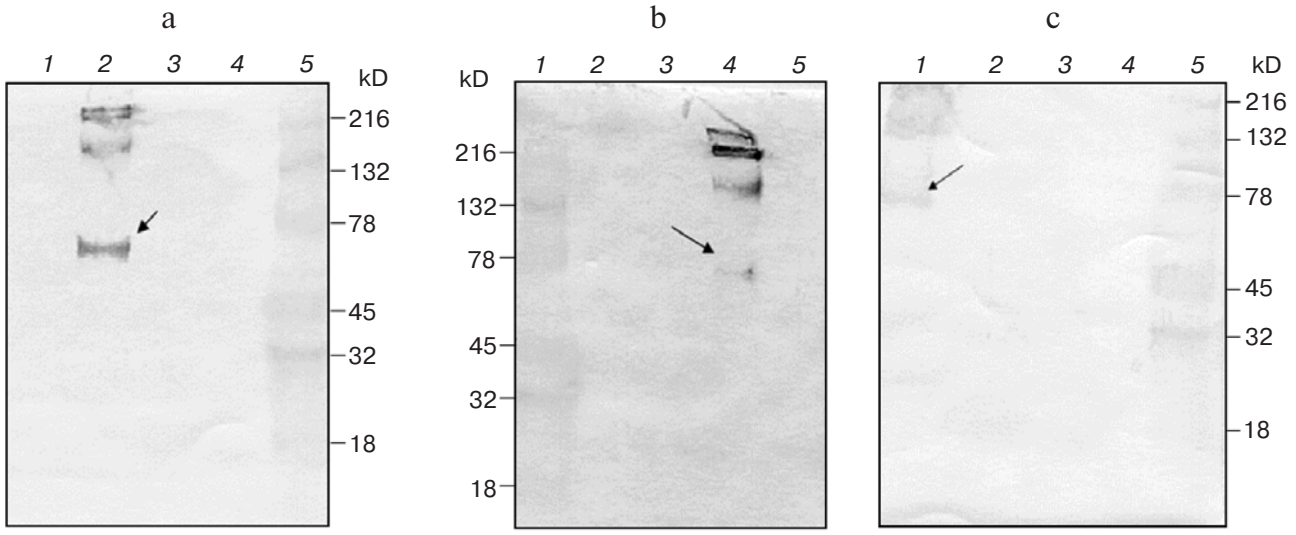


Fig. 4. Immunoblot analysis of the fusion proteins by GLUT antibodies. The SDS gel containing the indicated samples was transferred to a nitrocellulose membrane and subjected to Western analysis using the GLUT antibodies as the primary antibodies: a) GLUT1 antibody; b) GLUT2 antibody; c) GLUT3 antibody. a) Lanes: *1*) GST; *2*) GST–GLUT1; *3*) GST–GLUT2; *4*) GST–GLUT3; *5*) marker. b) Lanes: *1*) marker; *2*) GST; *3*) GST–GLUT1; *4*) GST–GLUT2; *5*) GST–GLUT3. c) Lanes: *1*) GST–GLUT3; *2*) GST–GLUT2; *3*) GST–GLUT1; *4*) GST; *5*) marker.

DISCUSSION

Eukaryotic membrane proteins play vital roles in the cell and are important drug targets. Over the past decade, numerous reports on the recombinant expression of integral membrane proteins have been published [2, 16, 17]. However, it is still very difficult to produce sufficient quantities of functional eukaryotic membrane proteins in heterologous expression systems. This provides a major challenge for structural investigations of eukaryotic membrane proteins.

It has been reported that eukaryotic hosts are generally better than prokaryotic hosts for producing functional eukaryotic membrane proteins [2, 18]. The advantages of using eukaryotic hosts to express eukaryotic membrane proteins are obvious; the systems for translation, targeting, insertion, and post-translational modifications are compatible, and the proteins are consequently more likely to be well-folded and active [19]. Some eukaryotic expression systems, such as animal cells, are likely to produce functional eukaryotic membrane proteins in their natural steric structures. However, these hosts are more difficult to handle than microorganisms, research conditions are strict, production efficiency is low, and the costs are high. To increase our knowledge of the function and structure of eukaryotic membrane proteins, more approaches for their overproduction need to be developed.

Yeast cells are well suited for producing heterologous eukaryotic proteins. They facilitate the post-translational processing of polypeptides, such as folding, phosphorylation, and glycosylation, and their cultivation methods are well established and cost-effective [20]. Among the yeasts, *S. pombe* is considered to be the most closely related to higher eukaryotes. *Schizosaccharomyces pombe* is comparable with eukaryotes in various aspects, such as the regulation of the cell cycle, transcription, chromosomal organization, and RNA splicing [10, 21-23]. Its post-translational protein modifications also appear to be similar to those in mammalian cells, such as glycosylation, phosphorylation, and acetylation [22, 24, 25]. Furthermore, recent evidence implies that, in contrast to *S. cerevisiae*, the quality control mechanism of glycoprotein folding in *S. pombe* is similar to that of mammalian cells [26].

Thus, the use of *S. pombe* as a host to express mammalian proteins is more likely to produce polypeptides that are similar to their native, biologically functional forms. The successful expression of human proteins in *S. pombe* has been reported; all of these human proteins were able to complement the respective biochemical function that was deficient in the mutant *S. pombe* [11, 27-30]. Even though the fission yeast is an experimental system for investigating problems of eukaryotic cell and molecular biology, it is highly regarded as a potential host for expressing eukaryotic membrane proteins because of its extensive available genetic information, and ease of genetic manipulation, the fission yeast is thought highly of a potential host for expressing eukaryotic membrane proteins [31].

Here, we tried this expression system to express and purify the GLUTs. Recent studies have provided new insight into the response of host cells to the expression of membrane proteins and the mechanism of membrane insertion. The successful overproduction of some membrane proteins has been shown to be linked to the avoidance of stress responses in the host cell. It was also

revealed that the translocon, which is the site of protein translocation and membrane insertion, determined whether a protein segment was integrated into the membrane [32]. In yeast, the unfolded protein response, a signaling mechanism that is triggered by the stress of protein expression, influences the overexpression of recombinant proteins and would be detrimental to host survival. Therefore, modulating overexpression levels to avoid this response is critical. In our study, repeated adjustments were made to the fission yeast cells growth and induction conditions. Even though we were unable to examine the response of the yeast, we were able to successfully express three human membrane proteins, GST–GLUT1, GST–GLUT2, and GST–GLUT3.

Our cell lysis buffer was a common buffer for membrane protein extraction, except for the addition of two neutral detergent mixtures, Triton X-100 and IGEPAL. Some cell membrane compartments show resistance toward certain detergents [33]; therefore, detergent mixtures in purification and crystallization protocols are sometimes more efficient than a single detergent [4, 34, 35]. As it turned out, the estimated yields of these proteins were quite large and exceeded our expectations. Approximately 300 μg of highly purified GST–GLUT1 and GST–GLUT3 fusion proteins and 100 μg of GST–GLUT2 protein were obtained from every 3 g of wet cell paste (1 liter of cell culture).

In this study, the human membrane proteins, GLUT1, GLUT2, and GLUT3, were overexpressed efficiently in the fission yeast. This result shows that *S. pombe* is a suitable system for the overproduction of human membrane proteins. We believe that this work will help alleviate the lack of structural knowledge concerning the human GLUTs. Currently, methods are being developed to further automate the purification process, and fermentation will be carried out on a larger scale. With the ability to produce and purify milligram amounts of GLUTs, we have begun extensive studies on these receptors to gain more information about their stability, folding, and interactions. We hope that our results will also contribute to the crystallization and high-resolution structure determination of these proteins.

This work was supported by the program “863” of the Ministry of Science and Technology of the People’s Republic of China (NO:2006AA02Z138), Natural Science Foundation of Chongqing of China (No. cstc, 2006BA5006, 2006BB1194), and Ministry of Science and Technology of China (973 project: 2003CB716601).

REFERENCES

1. Miroux, B., and Walker, J. E. (1996) *J. Mol. Biol.*, **260**, 289-298.
2. Sarramegna, V., Talmont, F., Demange, P., and Milon, A. (2003) *Cell Mol. Life Sci.*, **60**, 1529-1546.
3. Dobrovetsky, E., Menendez, J., Edwards, A. M., and Koth, C. M. (2007) *Methods*, **41**, 381-387.
4. Niegowski, D., Hedren, M., Nordlund, P., and Eshaghi, S. (2006) *Int. J. Biol. Macromol.*, **39**, 83-87.
5. Olson, A. L., and Pessin, J. E. (1996) *Annu. Rev. Nutr.*, **16**, 235-256.
6. Mueckler, M. (1994) *Eur. J. Biochem.*, **219**, 713-725.
7. Marger, M. D., and Saier, M. H. (1993) *Trends Biochem. Sci.*, **18**, 13-20.
8. Brown, G. K. (2000) *J. Inherit. Metab. Dis.*, **23**, 237-246.
9. Tohda, H., Okada, H., Giga-Hama, Y., Okayama, H., and Kumagai, H. (1994) *Gene*, **150**, 275-280.
10. Kaufer, N. F., Simanis, V., and Nurse, P. (1985) *Nature*, **318**, 78-80.
11. Giga-Hama, Y., Tohda, H., Okada, H., Owada, M. K., Okayama, H., and Kumagai, H. (1994) *Biotechnology*, **12**, 400-404.
12. Stoscheck, C. M. (1990) *Meth. Enzymol.*, **182**, 50-68.
13. Maundrell, K. (1989) *J. Biol. Chem.*, **265**, 10857-10864.
14. Lu, Q., Bauer, J. C., and Greener, A. (1997) *Gene*, **200**, 135-144.
15. Weiss, H. M., and Grisshammer, R. (2002) *Eur. J. Biochem.*, **269**, 82-92.
16. Tschantz, W. R., Pfeifer, N. D., Meade, C. L., Wang, L., Lanzetti, A., Kamath, A. V., Berlioz-Seux, F., and Hashim, M. F. (2008) *Prot. Exp. Purif.*, **57**, 163-171.
17. Jidenko, M., Lenoir, G., Fuentes, J. M., le Maire, M., and Jaxel, C. (2006) *Prot. Exp. Purif.*, **48**, 32-42.
18. Tate, C. G. (2001) *FEBS Lett.*, **504**, 94-98.
19. Kunji, E. R., Chan, K. W., Slotboom, D. J., Floyd, S., O’Connor, R., and Monne, M. (2005) *Curr. Opin. Biotechnol.*, **16**, 546-551.
20. Kjaerulff, S., and Jensen, M. R. (2005) *Biochem. Biophys. Res. Commun.*, **336**, 974-982.
21. Russell, P., and Nurse, P. (1986) *Cell*, **45**, 781-782.
22. Brys, R., Nelles, L., van der Schueren, E., Silvestre, N., Huylebroeck, D., and Remacle, J. E. (1998) *DNA Cell. Biol.*, **17**, 349-358.
23. Remacle, J. E., Albrecht, G., Brys, R., Braus, G. H., and Huylebroeck, D. (1997) *EMBO J.*, **16**, 5722-5729.
24. Moreno, S., Sanchez, Y., and Rodriguez, L. (1990) *Biochem. J.*, **267**, 697-702.
25. Chappell, T. G., and Warren, G. J. (1989) *Cell Biol.*, **109**, 2693-2702.
26. Parodi, A. J. (1999) *Biochim. Biophys. Acta*, **1426**, 287-295.
27. Bureik, M., Schiffler, B., Hiraoka, Y., Vogel, F., and Bernhardt, R. (2002) *Biochemistry*, **41**, 2311-2321.
28. Tabuchi, M., Yoshida, T., Takegawa, K., and Kishi, F. (1999) *Biochem. J.*, **344**, 211-219.
29. Tiede, A., Schubert, J., Nischan, C., Jensen, I., Westfall, B., Taron, C. H., Orlean, P., and Schmidt, R. E. (1998) *Biochem. J.*, **334**, 609-616.
30. Bayele, H. K., Eisenthal, R. S., and Towner, P. (2000) *J. Biol. Chem.*, **275**, 14217-14222.
31. Moreno, S., Klar, A., and Nurse, P. (1991) *Meth. Enzymol.*, **194**, 795-823.
32. Reinhard, G. (2006) *Curr. Opin. Biotechnol.*, **17**, 337-340.
33. Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A., and Simons, K. (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 5795-5800.
34. Koronakis, V., Sharff, A., Koronakis, E., Luisi, B., and Hughes, C. (2000) *Nature*, **40**, 5914-5919.
35. Lemieux, M. J., Song, J., Kim, M. J., Huang, Y., Villa, A., Auer, M., Li, X. D., and Wang, D. N. (2003) *Protein Sci.*, **12**, 2748-2756.