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Surface display of a glucose binding protein

Kaiming Ye[a,*], Sha Jin[b], Kelly Bratic[a], Jerome S. Schultz[a]

[a] *Department of Bioengineering, Center for Biotechnology and Bioengineering, University of Pittsburgh, 300 Technology Drive, Pittsburgh, PA 15219, USA*
[b] *Department of Molecular Genetics and Biochemistry, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15216, USA*

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Abstract

Glucose binding protein (GBP) from *Escherichia coli* has been widely used to develop minimally invasive glucose biosensors for diabetics. To develop a cell-based glucose biosensor, it is essential to functionally display GBP on the cell surface. In this study, we designed a molecular structure to display GBP on the outer membrane of *E. coli*. We fused GBP with the first nine N-terminal residues of Lpp (major *E. coli* lipoprotein) and the 46–150 residues of OmpA (an outer membrane protein of *E. coli*). With this molecular design, we have successfully displayed GBP on the surface of *E. coli*. Using FITC-conjugated Dextran, we demonstrated that glucose's binding sites of surface-displayed GBP were accessible to glucose. Furthermore, we showed that glucose transport in a GBP-deficient *E. coli* NM303 could be restored by displaying GBP on the surface of NM303. 0.51 h^{-1} of specific growth rate was attained for NM303/pESDG grown in M9 minimal medium supplemented with 2 g/l glucose, whereas no growth was observed for NM303 in the same medium. Both NM303 and NM303/pESDG grew in M9 medium supplemented with 1 mM of fucose. Because cell surface is an interface between intracellular and extracellular molecular events, this technique paves a way to develop cell-based glucose biosensors.

Keywords: Glucose binding protein; Cell surface protein display; Glucose binding assay; Cell surface engineering; *E. coli* surface protein display

1. Introduction

The functional display of a protein on cell surface opens up a new avenue for developing cell-based biosensors. The advantage by displaying a protein on cell surface is that the protein can be accessed directly by its affinitive ligands. Cell surface is an interface between intracellular and extracellular molecules. It consists of cell membrane and proteins that attach to the membrane. Different proteins are associated with the membrane in different ways. Many extend through the lipid bilayer called transmembrane proteins. Others are located entirely in the cytosol or entirely exposed at the external cell surface by a covalent linkage called anchor. A number of anchors have been identified and characterized [1,2]. For example, the glycosylphosphatidylinositol (GPI) anchor, which is added to the C-terminus of most cell membrane proteins, is a covalent linkage that localizes proteins on the outer membrane [3]. This anchoring mechanism has been utilized to display a variety of enzymes on cell surface [4,5]. We have previously developed a yeast surface reporting system in which a green fluorescent protein was flanked by a secretion signal sequence at its N-terminus and a GPI anchor at its C-terminus, respectively [6,7]. The secretion signal taken from the fungal glucoamylase precursor protein served as a targeting signal to direct EGFP across through the membrane and to localize on the cell surface. A variety of cell surface protein display systems have been recently developed as well. Francisco et al. [8] have developed an *E. coli* surface protein display system, in which a secretion signal and an anchor were tandem fused to N-terminus of a protein, by which the protein could be localized on the outer membrane of *E. coli*.

Herein, we report a novel approach to functionally display a glucose binding protein (GBP) on the surface of *E. coli*. GBP is a 32-kDa globular periplasmic binding protein that functions as a transporter for glucose in *E. coli* [9]. The protein possesses two distinct helical structural domains, each organized in a α/β folding motif involving the

* Corresponding author. Tel.: +1-412-383-7132; fax: +1-412-383-9710.
E-mail address: kaiming@pitt.edu (K. Ye).

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glucose binding region [10]. Like other periplasmic binding proteins, GBP undergoes a conformational change upon glucose binding, making it ideal for developing noninvasive glucose biosensors [11–14]. Display of GBP on the cell surface will allow for constructing cell-based glucose biosensors.

To display GBP on the surface of *E. coli*, we fused the protein with the first nine N-terminal residues of Lpp (major *E. coli* lipoprotein) and the residues 46–159 of OmpA. OmpA is an outer membrane protein with 325 amino acid residues [15]. It has been demonstrated that the region between the residues 154 and 180 of OmpA is crucial for localization of a protein [16]. The region between the residues 46 and 159 contains an export signal, directing the protein to the outer membrane [8]. The first nine residues of Lpp served as a targeting signal, mediating a proper localization of the protein on the outer membrane [17]. The goal of this study is to assess the accessibility of glucose binding sites of *E. coli* surface-displayed GBP to the glucose. Furthermore, we intend to determine whether glucose transport in a GBP-defective *E. coli* NM303 could be restored by displaying GBP on its surface.

2. Materials and methods

2.1. Bacteria and plasmids

E. coli DH5α (F$^-$ ϕ80d*lac*ZΔM15Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17(r_k^- m_k^+) *deo*R *thi*-1 *pho*A *sup*E44 λ$^-$ *gyr*A96 *rel*A1) was used for all experiments. DH5α was grown in the LB medium (Life Technology, NY, USA), supplemented with 100 μg/ml of ampicillin. To induce the expression of GBP on the surface of *E. coli*, 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) (Gold BioTechnology, MO, USA) was added to the medium when DH5α reached a mid-exponential growth phase (OD_{600} = 0.4–0.6). cDNA of GBP was cloned from *E. coli* K12 [11]. The leading signal peptide sequence and the start codon of GBP were deleted from its cDNA sequence. Two restriction enzyme sites, *Spe*I and *Kpn*I, were introduced into the PCR-amplified GBP at its 5′ and 3′ ends, respectively. The resultant plasmid was named pTAGBP. Plasmid pSD192 was kindly provided by Georgiou [8]. The fragment of Lpp-OmpA was amplified from pSD192. *Nco*I and *Spe*I sites were introduced at 5′ and 3′ ends of the Lpp-OmpA fragment, respectively. The Lpp-OmpA was subcloned into pTAGBP through the sites of *Nco*I and *Spe*I. The resultant plasmid was designated as pESDG. The fusion Lpp–OmpA–GBP gene was placed under immediate downstream of the promoter *trc* [18]. A repressor gene, *lac*O, was coupled with the promoter to provide a tight control of GBP expression [19]. To facilitate the detecting of surface-displayed GBP, we have fused a hexohistidine $(His)_6$ tag to the C-terminus of Lpp–OmpA–GBP.

2.2. Cell fractionation

A 2-ml portion of overnight culture of recombinant *E. coli* was inoculated into 100 ml of the LB medium supplemented with 100 μg/ml of ampicillin. *E. coli* were harvested 6 h postinduction with IPTG, washed twice in 20 mM Tris–HCl buffer (pH 7.5), and resuspended in 5 ml of B-PER protein extraction buffer (Pierce, IL, USA). 1 mM phenylmethylsulfony fluoride (PMSF) (Sigma, MO, USA) was added to the extraction buffer to stabilize the proteins during extraction. After 10 min incubation at room temperature, clear cell lysates were prepared by centrifuging the extraction solution for 15 min at 21,000 × *g* at 4 °C. The membrane fraction was collected by ultracentrifugation of the cell lysates for 1 h at 80,000 × *g* at 4 °C. The membranes were resuspended in 0.5 ml of 20 mM Tris–HCl buffer and used for Western blot.

2.3. SDS–PAGE

Protein electrophoresis was performed according to the methods of Laemmi [20]. The samples were mixed with 2× loading buffer (Sigma, MO, USA) and heated at 95 °C in a heater blocker (Fisher Science, PA, USA) for 5 min before loading onto a 10% of precast SDS–PAGE gel (Bio-Rad, CA, USA). Proteins were visualized by staining the gels with Coomassie Brilliant Blue R-250 and documented with the Kodak 1 D gel analysis system (Kodak, Tokyo, Japan).

2.4. Western blot

Proteins in each cell fraction were resolved on a 10% of polyacrylamide gel and transferred onto a nitrocellulose membrane (0.2 μm) (Bio-Rad, CA, USA) using a semidry electroblotter (Alltech, NY, USA), according to the protocol provided by the manufacturer. The blots were blocked for 1 h at room temperature in 5% nonfat powered milk in TBS (0.1% Tween 20 (pH 7.5), 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}12H_2O$ and 1.5 mM KH_2PO_4) and incubated with anti-His (C-term)-horseradish peroxidase (HRP) conjugate antibody (1:5000 diluted in the blocking buffer) (Pierce, IL, USA) for 1.5 h at room temperature. After washing in TBS buffer four times each for 5 min with gentle shaking, the blots were incubated in the chemiluminescence reagent (Pierce, IL, USA) for 1 min at room temperature and visualized by the 1D gel analysis system.

2.5. Flow cytometry and fluorescence microscopy

The recombinant *E. coli* were harvested at mid-exponential growth phase and washed twice with 10 mM PBS buffer. After washing, the bacteria were incubated in PBS buffer for 20 min at 37 °C to consume the intracellular glucose. After further washing with 10 mM PBS supplemented with 1 mM $CaCl_2$ (binding buffer), ∼10^6 bacteria were resuspended in 100 μl of FITC-Dextran (10 μM dissolved in the binding buffer) (Sigma, MO, USA). After incubation for 45 min at

room temperature, the bacteria were pelleted and washed twice with the binding buffer. A 10-μl portion of the bacteria was loaded onto a glass slide and examined under a fluorescence microscope (Olympus IX-70, Japan) equipped with a FITC filter (Chroma, VT, USA) and a CCD camera (Qimage, Canada). The surface-displayed GBP was also detected by FACScan (Becton Dickinson, MA, USA). After incubating the FITC-Dextran with the recombinant *E. coli* at 4 °C, 10^6 bacteria were washed three times in cold binding buffer, and then resuspended in 0.5 ml of cold binding buffer. The samples were applied immediately to the flow cytometric analysis using FACScan.

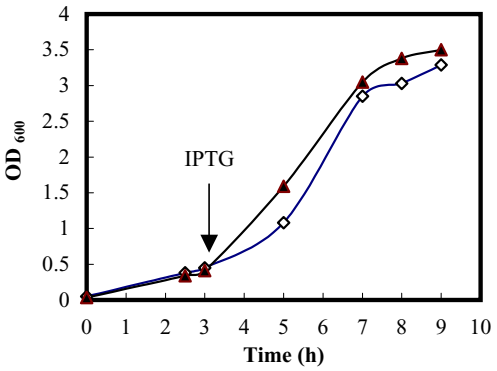

Fig. 1. The effect of the surface-displayed GBP on the cell growth. Symbols: (▲), wild type *E. coli* DH5α; (◇), *E. coli* DH5α/pESDG.

3. Results and discussion

3.1. Display of GBP on the surface of E. coli

To functionally display GBP on cell surface of *E. coli*, we have deleted the leading peptide sequence of GBP, resulting in a truncated GBP consisting of 309 amino acid residues. We fused the Lpp-OmpA composed of a secretion signal and an anchor sequence to the N-terminus of GBP. The plasmid pESDG encoding the fusion protein Lpp–OmpA–GBP was transformed into *E. coli* DH5α and used to display GBP on the surface of *E. coli*. To determine possible inhibition of the surface displaying of GBP on the growth of *E. coli*, we monitored the growth rate of the recombinant DH5α/pESDG. We found that the growth of the recombinant *E. coli* was slightly inhibited by the expression of the fusion protein Lpp–OmpA–GBP (Fig. 1). Cell density of the recombinant DH5α/pESDG reached 3.8 of OD_{600} 6 h postinduction, whereas wild-type DH5α reached 4.3 of OD_{600}, indicating no significant effect of the surface displaying of GBP on the growth of *E. coli*.

In the next experiment, we determined the distribution of GBP in the recombinant DH5α/pESDG by cell fractionation. The fusion protein Lpp–OmpA–GBP in each cell fraction was detected by SDS–PAGE. The molecular weight of Lpp–OmpA–GBP including $(His)_6$ tag was estimated to have 52 kDa. A HRP-conjugated anti-polyhistidine antibody was employed to probe Lpp–OmpA–GBP in the Western blot. Wild-type DH5α served as a control. As shown in Fig. 2, Lpp–OmpA–GBP was detected in the membrane fraction of the recombinant DH5α/pESDG, but neither in the cell-free extraction fraction nor in the medium fraction of DH5α/pESDG. This result suggests that GBP was delivered to the membrane without proteolytic degradation during export. Furthermore, no Lpp–OmpA–GBP was found in the medium fraction, indicating that no secretion of the fusion protein occurred during export. Taken together, we demonstrated that GBP was exported and localized at the surface of *E. coli*.

3.2. Affinity detection of E. coli surface-displayed GBP

The affinity of surface-displayed GBP for the glucose was determined by both fluorescence microscopy and flow cytometric analysis. We used FITC-conjugated Dextran for the binding assay. Dextran has internal pendent glucose residues that can serve as receptors for GBP. The binding of FITC-Dextran to the surface-displayed GBP made *E. coli* visible under a fluorescence microscope and detectable by a flow cytometer. Because GBP is a periplasmic binding protein that serves as a glucose transporter, it expresses at a low level in *E. coli*. To eliminate the interference of the

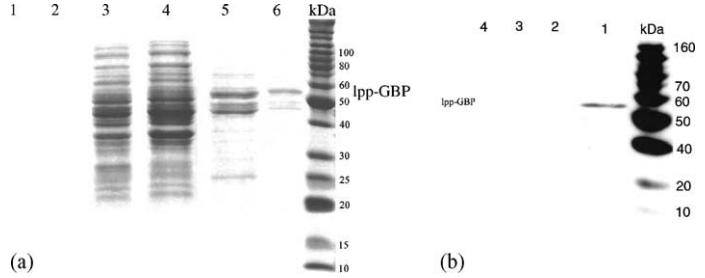

Fig. 2. Detection of *E. coli* surface-displayed GBP. (a) SDS–PAGE of each cell fraction of DH5α/pESDG. Lanes: 1, the medium fraction of DH5α; 2, the medium fraction of DH5α/pESDG; 3, the cell lysate of DH5α; 4, the cell lysate of DH5α/pESDG; 5, the cell membrane fraction of DH5α; 6, the cell membrane fraction of DH5α/pESDG. Symbol: lpp-GBP, the Lpp–OmpA–GBP fusion protein. (b) Western blot of each cell fraction of DH5α/pESDG. Lanes: 1, the cell membrane fraction of DH5α/pESDG; 2, the cell membrane fraction of DH5α; 3, the cell lysate of DH5α/pESDG; 4, the cell lysate of DH5α. The anti-His HRP conjugate antibody was used to detect C-terminally $(His)_6$-tagged GBP in each cell fraction.

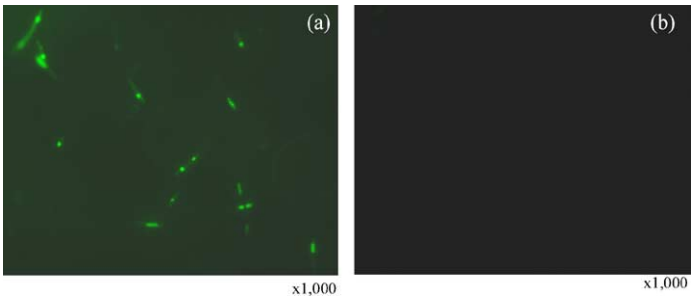

Fig. 3. Affinity assay of the surface-displayed GBP using FITC-Dextran. (a) NM303/pESDG; (b) NM303.

spontaneous expression of GBP, we used a GBP-deficient *E. coli* NM303 (a kind gift from Dr. Lakowicz), which GBP gene was knocked out. The plasmid pESDG was transformed into NM303. The resultant NM303/pESDG was grown in the LB medium supplemented with 1 mM of fucose. 9×10^7 NM303/pESDG were collected 6 h postinduction and incubated with FITC-Dextran. The binding of FITC-Dextran to *E. coli* surface-displayed GBP was visually detected with a fluorescence microscope. Because of the binding of FITC-Dextran to GBP, NM303/pESDG became fluorescent and detectable under a fluorescence microscope (Fig. 3a). Conversely, none of NM303 was labeled by FITC-Detran (Fig. 3b). This experiment verified that the glucose's binding sites of the surface-displayed GBP were accessible to the glucose.

To perform a flow cytometric analysis, $\sim 1 \times 10^6$ *E. coli* were incubated with FITC-Dextran (10^{-5} M) for 45 min at 4 °C. After washing, *E. coli* were resuspended in 1 ml of the binding buffer and applied to the flow cytometry immediately. As presented in Fig. 4, the fluorescence intensity of NM303/pESDG was clearly distinguishable from the intrinsic signal of wild-type NM303 (M2 region in Fig. 4b). The background signals detected in NM303 might be the result of nonspecific binding of FITC-Dextran to the bacteria. About 30.22% (M2 region in Fig. 4a) of NM303/pESDG were labeled by FITC-Dextran and emitted a bright fluorescence from the surface of *E. coli*. We interpreted that the low ratio of the labeled over unlabeled NM303/pESDG was possibly due to a poor expression of GBP on the surface of *E. coli*. Furthermore, we found that the expression level of GBP on the surface of *E. coli* varied significantly. As shown in Fig. 4a, the fluorescence intensity of the labeled *E. coli* had a wide distribution, indicating the difference in the expression level of GBP on the surface of *E. coli*.

3.3. The restoring of glucose transport in GBP-deficient *E. coli* by displaying GBP on its surface

To investigate whether we could restore glucose transport in a GBP-deficient *E. coli* by displaying GBP on its surface, we used NM303. NM303 cannot utilize glucose as a sole carbon source as its GBP gene was knocked out. To grow NM303 in M9 minimal medium, supplementing the medium with fucose is essential. We documented the growth of NM303, as well as the growth of NM303/pESDG, in M9 medium supplemented with or without glucose. As predicted, NM303 did not grow in M9 medium supplemented with 2 g/l of glucose (Fig. 5). Nevertheless, both NM303 and NM303/pESDG grew in M9 medium supplemented with fucose (1 mM). However, the growth rate was very low because the fucose was not a favor of carbon source for *E. coli*. The specific growth rate of NM303 at the exponential growth phase was about 0.239 h^{-1}, whereas it was 0.236 h^{-1} for NM303/pESDG. Of particular interest, the

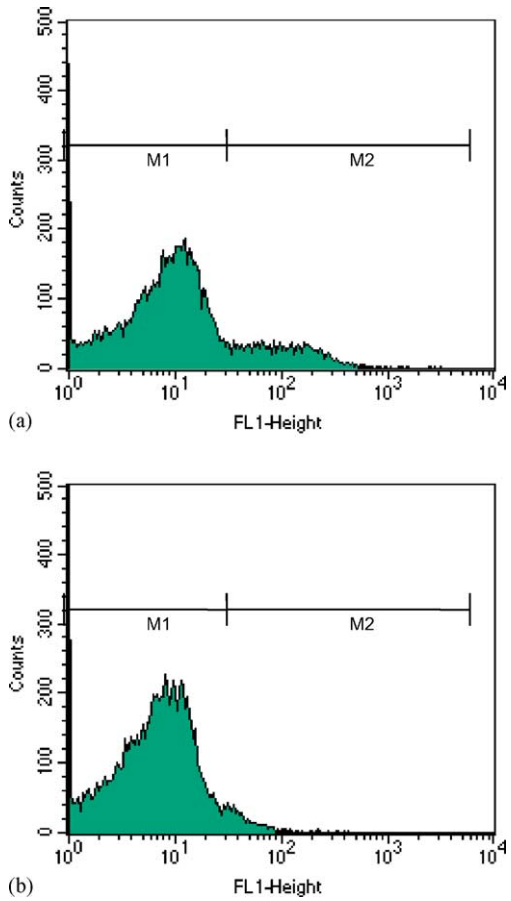

Fig. 4. Flow cytometric analysis of the surface-displayed GBP. Data from 10,000 events are shown. (a) NM303/pESDG; (b) NM303. Symbol: FL1-Height, fluorescence intensity of FITC-Dextran; M1: FITC-Dextran unlabeled *E. coli*; M2: FITC-Dextran labeled *E. coli*.

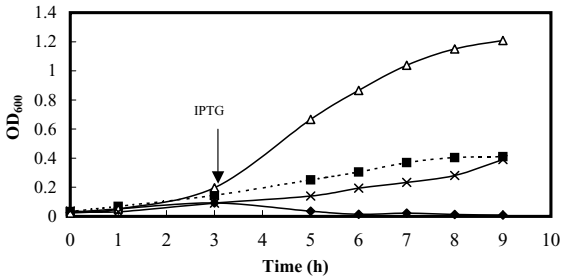

Fig. 5. Restoration of glucose transport in NM 303 by displaying GBP on its surface. All the bacteria were grown in M9 minimal medium supplemented with or without glucose. IPTG (1 mM) was added to the M9 medium 3 h postinoculation to induce the expression of GBP on the surface. Symbols: (△), NM303/pESDG in M9 medium supplemented with 2 g/l of glucose; (×), NM303/pESDG in M9 medium supplemented with 0.164 g/l of fucose; (■), NM303 in M9 medium supplemented with 0.164 g/l of fucose; (◆), NM303 in M9 medium supplemented with 2 g/l of glucose.

growth of NM303/pESDG became faster in M9 medium supplemented with glucose after induction with IPTG. Its specific growth rate was increased up to 0.51 h^{-1}. It was almost two times higher than that of NM303 and NM303/pESDG in M9 medium supplemented with fucose. Rapid growth of NM303/pESDG in M9 glucose medium supplemented with glucose indicates that the glucose transport in NM303/pESDG was restored by displaying GBP on the surface of *E. coli*.

4. Conclusion

In this study, we demonstrated that GBP could be expressed functionally on the surface of *E. coli*, and that the displayed GBP retained its high affinity to glucose. The flow cytometric analysis showed that the expression level of GBP on the surface of *E. coli* varied significantly. Moreover, the FITC-Dextran-labeled NM303/pESDG could be clearly discriminated from the intrinsic background signal of the control NM303. This result suggests that FACS may be used for screening a surface-displayed combinatorial library of GBP for a desired affinity for the glucose. The studies on the growth of NM303 and NM303/pESDG in M9 medium supplemented with different carbon sources revealed that the surface-displayed GBP retained not only its high affinity for glucose but also its glucose transport function. We observed that the metabolism of glucose in NM303/pESDG was restored after the expression of GBP on the surface of *E. coli*. These preliminary experimental data strongly imply that the function of glucose transport could be genetically introduced into *E. coli* by displaying GBP on its surface. Because the cell surface is a key interface between intracellular and extracellular molecular events, this discovery may be important for altering or engineering the cell surface for the studies of glucose metabolism.

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