A Detection Method for GLUT4 Exocytosis Based on Spontaneous Split Luciferase Complementation

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Glucose transporter 4 (GLUT4) is an insulin-regulated glucose transporter, which is vital for blood glucose homeostasis. To clarify the physiological roles of GLUT4, quantitative measurement of GLUT4 exocytosis is indispensable. Herein, we show a rapid detection system for GLUT4 on the cell surface using spontaneous split-luciferase reconstitution. Upon insulin-induced GLUT4 exocytosis, GLUT4 was exposed outside, where luciferase is reconstituted and emitted luminescence. Pretreatment with inhibitors reduced the insulin-induced signal elevation. The results indicate that the developed method is applicable to high-throughput analysis on GLUT4 trafficking, which will greatly accelerate comprehensive research on the physiological roles of GLUT4.

Keywords GLUT4, exocytosis, bioluminescence, luciferase, NanoLuc

(Received June 19, 2019; Accepted June 28, 2019; Advance Publication Released Online by J-STAGE July 5, 2019)

Introduction

Glucose transporter 4 (GLUT4) is an insulin-regulated glucose transporter, which is vital for glucose uptake in response to insulin stimulation. Upon insulin binding to its receptor on plasma membrane, downstream molecules such as phosphoinositide 3-kinase (PI3K) and Rab GTPases are activated. The signaling triggers translocation of intracellular GLUT4-containing vesicles to the cell surface, where glucose uptake takes place. The lack of insulin-stimulated GLUT4 translocation commonly associated with type 2 diabetes mellitus results in a wide array of physiological damage. Thus, it is important to unveil the molecular mechanisms of insulin-stimulated GLUT4 translocation, which requires quantitative measurement of GLUT4 trafficking.

Conventionally, GLUT4 on the cell surface is detected by immunostaining, in which HA epitope tag is inserted in the exofacial loop. HA-tagged GLUT4 on the cell surface is labeled with anti-HA antibody without membrane permeabilization. The labeled GLUT4 is detected by fluorescence microscope or by flow cytometry. Although the method successfully detects insulin-induced GLUT4 translocation, it requires multiple experimental steps to label GLUT4, which hampers rapid and simultaneous analysis. Therefore, there is a need to develop a novel method that allows for detection of GLUT4 translocation under various conditions in a high-throughput manner.

Luciferase, an enzyme that generates bioluminescence upon substrate catalysis, has been used to monitor protein activities or interactions quantitatively. NanoLuc luciferase (NLuc) has been used as a quantitative luminescent peptide tag based on split luciferase complementation. In the system, NLuc is split into two fragments, which lose the ability of luminescence. Upon spontaneous interaction ($K_D = 700$ pM), the NLuc fragments reconstitute into native structure of NLuc and emit luminescence, which enables rapid detection of NLucC-tagged proteins by adding NLucN and the substrate.

Herein, we demonstrate a rapid detection system for GLUT4 on the cell surface using spontaneous luciferase reconstitution (Fig. 1a). To monitor GLUT4 on the plasma membrane, a quantitative luminescent tag NLucC was inserted into the exofacial loop of GLUT4, named NLucC-GLUT4, and NLucN was added into a culture medium. Upon insulin stimulation, NLucC-GLUT4 translocated to the plasma membrane and the inserted NLucC was exposed outside, where both NLuc fragments spontaneously interacted and emitted luminescence. Pretreatment with inhibitors reduced the insulin-induced signal elevation. The developed method is applicable to high throughput screening for GLUT4 exocytosis inhibitors, which will greatly accelerate comprehensive research on mechanisms of GLUT4 trafficking and pathological relationship with diabetes.

Experimental

Cell culture, transfection, and infection

Human embryonic kidney (HEK293T) cells and Plat-E cells were maintained in DMEM (Nacalai Tesque Inc.) supplemented with 10% FBS (Thermo Fisher Scientific Inc.) at 37°C in 5% CO₂. For 3T3-L1 mouse fibroblasts, the medium was changed to DMEM supplemented with 10% DBS (Thermo Fisher Scientific Inc.). For 3T3-L1 cell differentiation, the medium was changed to DMEM high glucose, 10% FBS, 0.2 μM insulin (Sigma-Aldrich), 0.25 μM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine, two days after confluency (Day 0). On Day 2, the medium was changed to DMEM high glucose, 10% FBS, 0.2 μM insulin. On Day 4, the medium was replaced with the 10% FBS DMEM and was refreshed every two days. The assay was conducted on Days 8 to 12.

HEK293T cells were transiently transfected with TransIT-LT1 (TaKaRa Bio Inc.) according to the manufacturer’s protocol.
For retroviral production, Plat-E cells were transfected with TransIT-LT1. The produced viral particles were added to the medium culturing 3T3-L1, and 2 μg mL⁻¹ puromycin was added thereafter (InvivoGen).

**Immunocytochemistry**

Cells were serum-starved for 2 h at 37°C in low serum medium (DMEM supplemented with 0.1% FBS). After insulin stimulation (100 nM) for 30 min, cells were fixed with 4% paraformaldehyde. The cells were incubated with 2% bovine serum albumin, then reacted with the monoclonal anti-HA antibody conjugated with Alexa 488 (1:800, Cell Signaling Technology). Images were acquired using a confocal fluorescent microscope (IX-81 FV-1000D; Olympus Corp.).

**Luminescent measurement**

Transfected HEK293T cells were incubated with DMEM containing NLucN (50 nM) and NLuc-substrate furimazine (1:1000, Promega Corp.). For measurement with NLucC-GLUT4, 3T3-L1 cells were serum-starved for 2 h at 37°C in low serum medium (DMEM supplemented with 0.5% FBS). The cell medium was exchanged to DMEM containing LgBit (1:100, Promega Corp.) and furimazine (1:1000). After insulin stimulation, the cells were incubated for 30 min at 37°C. Luminescence was measured with a plate reader (TriStar930; Berthold Technologies), with a counting time of 1 s.

For luminescence imaging, the cell medium was exchanged to DMEM containing LgBit (1:400, Promega Corp.) and furimazine (1:200). After insulin stimulation, the cells were observed using an inverted fluorescence microscope (IX-81; Olympus Corp.) with a 20x objective lens (UPLSAPO20XO; Olympus Corp.). Luminescence images were acquired with a CCD camera (ImagEM; Hamamatsu Photonics) cooled to -70°C.

**Results and Discussion**

Spontaneous split NLuc reconstitution outside the cells

NLuc was split into two fragments (NLucN and NLucC between residues 156 and 157) and several mutations were inserted according to the previous report.³ To examine whether spontaneous NLuc complementation occurs outside the cell,
extracellularly-exposed NLucC molecule (NLucC-outside) and intracellularly retained NLucC molecule (NLucC-inside) were prepared (Fig. S1, Supporting Information). To generate NLucC-outside, the N-terminus of NLucC was fused with a signal peptide derived from CD59. The C-terminus of NLucC was fused with a glycosylphosphatidylinositol (GPI) anchor. NLucC-outside and NLucC-inside were expressed in HEK293T cells and their localization was analyzed using a confocal fluorescence microscope (Fig. S2a). We found that NLucC-outside was expressed on the plasma membrane, whereas NLucC-inside in cytosol. The transfected HEK293T cells were incubated with induction medium containing NLucN and furimazine. Luminescence measurement revealed that the cells expressing NLucC-outside emitted luminescence 108-fold higher than that from cells without transfection (Fig. S2b).

In contrast, cells expressing NLucC-inside did not yield luminescence, demonstrating that spontaneous reconstitution between NLucN and NLucC does not occur across the plasma membrane. Therefore, extracellularly-exposed NLucC is detected by bioluminescence, which is generated by spontaneous interaction with NLucN added into the cell culture medium.

**NLucC insertion into GLUT4 without affecting its insulin-induced trafficking**

In conventional immunocytochemistry analysis, the HA epitope tag was inserted between amino acid residues 67 and 68 located in the exofacial loop of GLUT4 (HA-GLUT4; Fig. S1). To detect GLUT4 on the cell surface, NLucC was inserted just after the HA tag in HA-GLUT4 (NLucC-GLUT4). 3T3-L1 adipocytes expressing HA-GLUT4 or NLucC-GLUT4 were observed under a confocal fluorescence microscope (Fig. S3a). The expressed molecules of HA-GLUT4 and NLucC-GLUT4 retained mostly in the cytoplasm, which was consistent with the previous reports. To detect GLUT4-containing molecules on the cell surface, 3T3-L1 adipocytes expressing NLucC-GLUT4 were immunostained with anti-HA antibody without membrane permeabilization. Confocal fluorescence microscope analysis revealed that there was almost no NLucC-GLUT4 on the plasma membrane (Fig. S3b). To examine NLucC-GLUT4 exocytosis upon insulin stimulation, the cells expressing NLucC-GLUT4 were stimulated with 100 nM insulin for 30 min. Immunocytochemistry clearly detected GLUT4 on the cell surface, indicating that NLucC-GLUT4 translocated to the plasma membrane upon insulin stimulation (Fig. S3b). Consequently, we concluded that NLucC insertion into the GLUT4 does not perturb native GLUT4 trafficking.

**Bioluminescence detection of GLUT4 on the cell surface using split-NLuc reconstitution**

To examine whether split-NLuc reconstitution allows for detecting GLUT4 on the cell surface, 3T3-L1 adipocytes expressing NLucC-GLUT4 were incubated with NLucN and furimazine. Luminescence measurement revealed that cells expressing NLucC-GLUT4 emitted high luminescence compared to control cells (Fig. 1b). Insulin stimulation triggered an increase in the luminescence in the cells expressing NLucC-GLUT4. Moreover, luminescence intensity versus insulin concentration yielded a typical sigmoidal curve (IC50: 8.5 nM, Fig. 1c). The bioluminescence microscopy analysis detected luminescence mainly emitted from the plasma membrane (Fig. S4). Collectively, the results demonstrate that GLUT4 translocation upon different doses of insulin can be quantitatively analyzed with the present method based on split-NLuc reconstitution.

Inhibitor screening for insulin-induced GLUT4 translocation is important for unveiling the molecular mechanisms. To investigate whether inhibitor treatment affects the insulin-induced luminescence elevation, 3T3-L1 adipocytes expressing NLucC-GLUT4 were pretreated with different inhibitors, then stimulated with insulin. Luminescence measurements revealed that treatment with 500 nM wortmannin, a PI3-kinase inhibitor, or 70 μM trifluoperazine, a calmodulin inhibitor, reduced the insulin-triggered luminescence elevation (Fig. 1d). The luminescence intensity versus trifluoperazine concentration yielded a sigmoid curve (IC50: 15.6 μM, Fig. 1e). In contrast, pretreatment with 10 μM SB203580, which attenuates insulin-stimulated glucose uptake without altering GLUT4 translocation, did not affect luminescence signal. Taken altogether, the present system is able to quantitatively evaluate the inhibitory effect of specific molecules on insulin-induced GLUT4 translocation.

**Conclusions**

In this study, GLUT4 on the cell surface was detected by extracellular spontaneous split-luciferase reconstitution. We confirmed that NLucC on the cell surface was detected by luminescence upon extracellular spontaneous reconstitution with NLucN. NLucC-GLUT4 on the cell surface was detected by split-NLuc reconstitution on the plasma membrane. Inhibitor treatment reduced the insulin-induced signal elevation. The developed method based on split luciferase reconstitution is useful for high throughput screening for GLUT4 trafficking inhibitors. This principle is generally applicable to investigate translocation of other membrane proteins. In the future, the present method will greatly lead to the deeper understanding of GLUT4 trafficking mechanisms and pathological effects in diabetes.

**Acknowledgements**

This work was supported by CREST (JPMICR1752 to T. O.) from the Japan Science and Technology (JST), and the Japan Society for the Promotion of Science (JSPS) KAKENHI (Grants-in-Aid for Scientific Research (S) 26220805, A 19H00900, and Innovative Areas JP 19H04952 to T. O.).

**Supporting Information**

Supplementary data associated with this article can be found, in the online version. These materials are available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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