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Identification of Glypican3 as a novel GLUT4-binding protein

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ABSTRACT

Insulin stimulates glucose uptake in fat and muscle primarily by stimulating the translocation of vesicles containing facilitative glucose transporters, GLUT4, from intracellular compartments to the plasma membrane. Although cell surface externalization of GLUT4 is critical for glucose transport, the mechanism regulating cell surface GLUT4 remains unknown. Using a yeast two-hybrid screening system, we have screened GLUT4-binding proteins, and identified a novel glycosyl phosphatidyl inositol (GPI)-linked proteoglycan, Glypican3 (GPC3). We confirmed their interaction using immunoprecipitation and a GST pull-down assay. We also revealed that GPC3 and GLUT4 to co-localized at the plasma membrane, using immunofluorescent microscopy. Furthermore, we observed that glucose uptake in GPC3-overexpressing adipocytes was increased by 30% as compared to control cells. These findings suggest that GPC3 may play roles in glucose transport through GLUT4.

Insulin stimulation of glucose uptake into skeletal muscle and adipose tissue is achieved via the translocation of intracellular-sequestered GLUT4 protein to the cell surface membrane [1,2]. On the plasma membrane, GLUT4 proteins, responding to insulin stimulation, remain externalized for a certain period of time and facilitate glucose transport. Although cell surface externalization of GLUT4 is critical for glucose transport, the mechanism regulating cell surface GLUT4 remains largely unknown. We speculated that it would require a protein capable of interacting with the glucose transporter.

Over the past decade, several GLUT4-binding proteins, such as mUbc9 [3], TUG [4], DAXX [5], L-3-hydroxyacyl-CoA dehydrogenase [6], and carboxyl esterase [7], have been identified using the C-terminus region of GLUT4 as bait in either a two-hybrid system or an immobilized GST fusion protein pull-down experiment. For example, mUbc9 was demonstrated to regulate transporter degradation [3], whereas TUG was shown to modulate GLUT4 distribution [4]. However, the functions of other GLUT4-binding proteins are not fully understood. We speculated that a change in the three-dimensional structure of the GLUT4 partial sequence has made it difficult to identify and analyze functional GLUT4 binding proteins. Thus, we used full-length GLUT4 for screening, and thereby identified GPC3 as a GLUT4-binding protein.

GPC3 is one of the heparan sulfate proteoglycans that are anchored to the cell membrane by a glycosyl-phosphatidylinositol protein [8]. This family of proteins was shown to be related to morphogenesis and GPC3 was originally reported to be a negative regulator of cell proliferation as well as the progression of malignant tumors [9–13]. Herein, we report GPC3 as a newly identified GLUT4-binding protein. This is the first report describing a protein that acts directly on GLUT4 molecules at the plasma membrane.

Materials and methods

Antibodies. Mouse monoclonal GPC3 antibody was provided by Dr. Hiroyuki Aburatani (University of Tokyo, Japan). The following antibodies were used: anti-GLUT4 rabbit and goat, anti-GST (Santa Cruz Biotechnology, CA); anti-FLAG (M2) (Sigma); anti-Myc (9E10) (Convance, CA) and fluorescent-conjugated and horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno-Research Laboratories).

Constructs. Mouse GPC3 cDNA was purchased from Open Biosystems (Huntsville, AL). Wild-type GPC3 was subcloned into a pGEX-6P1 (GE Healthcare Biosciences) vector. FLAG-tagged GPC3 and 4×Myc-tagged GLUT4-eGFP was subcloned into a pcDNA3 (Invitrogen) vector. All chemically synthesized and PCR-derived DNA sequences were verified by DNA sequencing.

Preparation of recombinant adenovirus vectors. Recombinant adenovirus encoding eGFP or FLAG-tagged GPC3 was constructed using the AdEasy adenovirus vector system according to the manufacturer's instructions (Startagene). All amplified viruses were stored at −80 °C. 3T3-L1 adipocytes were infected with recombinant adenovirus vectors encoding eGFP and FLAG-tagged GPC3 at a multiplicity of infection (m.o.i.) of 50.

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Cell culture. 3T3-L1 fibroblasts were grown in DMEM with 10% fetal bovine serum (FBS) at 37 °C. The cells (3–5 days post-confluent) were differentiated into adipocytes by incubation in the same DMEM containing 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, and 4 μg/ml insulin for 3 days, and then grown in DMEM with 10% FBS for an additional 3–6 days. Human hepatocellular carcinoma cell line HepG2 cells were cultured in Eagle's minimum essential medium (EMEM) with 10% FBS in at 37 °C.

Primary culture of mouse hepatocytes. Mouse hepatocytes were prepared from 5-month male C57BL/6J mice as described previously [14]. Isolated cells were seeded onto coverslips and allowed to recover for 24 h.

Yeast two-hybrid screening. The MATCHMAKER LexA Two-hybrid System (Clontech) was used for identification of GLUT4-binding proteins. As bait for screening, the vector pLexA-GLUT4 expressing a fusion protein composed of full-length rat GLUT4 linked to the DNA-binding domain was constructed. A rat adipocyte cDNA library already cloned into the pB42AD vector was obtained from Origene Technologies (Rockville, MD). Approximately 300 colonies showed activation of the yeast reporter gene, and 15 colonies showing dependence on the LexA-GLUT4 fusion protein for activation of the reporter gene were selected. Plasmids from positive clones were subsequently isolated from the yeast, transferred to E. coli, and sequenced. Full-length cDNA was obtained by 5′RACE (Rapid Amplification of cDNA End) using a kit (Clontech) and the GenBank/NCBI databases were screened for similar sequences using BLAST Search.

In vitro GST pull-down assay. GST fusion proteins of full-length GPC3 and GST alone were purified according to the manufacturer's instructions. GLUT4 protein was generated from Myc-tagged GLUT4-eGFP (four-Myc epitope-tags in the first exofacial loop and eGFP in the C-terminus) transfected 293 cells and further purified using anti-Myc antibody. These GST fusion proteins and purified 4×Myc-tagged GLUT4-eGFP were mixed in PBS and incubated at 4 °C for 4 h. The proteins were then pulled down with glutathione–sepharose beads (GE Healthcare Biosciences).

Immunoprecipitation and immunoblotting. Cells were lysed in lysis buffer [20 mM Hepes (pH 7.2), 100 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM sodium vanadate, 1 mM benzamidine, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, and 1 mM DTT] and the protein concentration was measured with BCA protein assay reagent (Pierce, Rockford, IL). For immunoprecipitation, the cell lysate was pre-incubated with protein-G Sepharose at 4 °C for 30 min to remove non-specific bound proteins. Then, samples were incubated with primary antibody at 4 °C for 8–12 h followed by incubation with protein-G Sepharose. Lysates and immunoprecipitates were resolved by SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Biosciences). The membranes were incubated with appropriate antibodies.

Immunofluorescence microscopy. 3T3-L1 adipocytes, HepG2 cells, and primary hepatocytes were seeded onto coverslips and allowed to recover for 24–48 h. 3T3-L1 adipocytes were serum-starved for 4 h in DMEM, followed by incubation with or without 100 nM of insulin for 15 min at 37 °C. Then, all of the cells were fixed with 3.7% formaldehyde in PBS and permeabilized with buffer A (0.5% Triton X-100 and 1% FBS in PBS) for 15 min, and finally incubated for 2 h with primary antibodies at room temperature. The cells were washed and incubated with an appropriate secondary antibody, or rhodamine-conjugated wheat germ agglutinin (Molecular Probes, Inc.) as a counter staining of cell membrane and Golgi system. The coverslips were washed thoroughly and mounted on glass slides. Immunostained cells were observed at room temperature with a laser-scanning confocal microscope (LSM5 PASCAL; Carl Zeiss Inc.).

Plasma membrane sheet assay. Cell surface protein was assayed using plasma membrane lawns as described previously [15]. The cells were subsequently swollen using hypotonic buffer and sonicated to generate a lawn of plasma membrane fragments. The membranes were immunostained with anti-GPC3 and anti-GLUT4 antibody. These cells were observed by laser confocal microscopy.

2-Deoxy-glucose uptake. Differentiated adipocytes were prepared in 24-well plates. Cells were infected with the recombinant adenoviruses. Two days thereafter, the cells were serum-starved for 2 h at 37 °C in Krebs–Ringer phosphate buffer (130 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 10 mM Na_2HPO_4, pH 7.4). The cells were then stimulated with or without 100 nM of insulin for 15 min, and deoxy-glucose uptake was determined by 2-deoxy-D-[2,6^{3}H] glucose incorporation.

Results and discussion

Identification of GLUT4-binding proteins

Regulation of glucose uptake in muscle and adipose tissues by insulin is important for proper maintenance of blood glucose. This hormone stimulates translocation of the GLUT4 glucose transporter from the intracellular membrane to the cell surface. After translocation to the plasma membrane, GLUT4 remains on the cell surface temporarily and facilitates glucose transport. However, the mechanism regulating cell surface GLUT4 is still largely unknown. We hypothesized that it requires protein–protein interactions at the plasma membrane. We used the yeast two-hybrid screening system to identify proteins that interact physically with full-length GLUT4. As bait, we used full-length rat GLUT4 cDNA and, as prey, a rat adipose tissue cDNA library. After the first screening, we obtained more than 300 colonies. After a second screening, 15 colonies remained positive. Ultimately, nine colonies were left. Full-length cDNAs were obtained by 5′RACE. One of them was 100% identical to GPC3 [16], which was originally cloned as OCI-5, a GPI anchored membrane protein [17]. Since we also obtained mUbc9 [3], which was previously identified using the same method, our experimental procedure was thought to have worked correctly. The GPC3 sequence obtained with the yeast two-hybrid system was comprised of the residues from 521 to 597. In order to confirm the interaction between GLUT4 and GPC3, we employed the yeast two-hybrid system again; using full-length GLUT4 cDNA and full-length GPC3 cDNA as the bait and prey, respectively. We confirmed the LacZ signal indicating a direct interaction between GPC3 and GLUT4 (Fig. 1A). Next, we determined GPC3 protein expression using Western blot analysis. As shown in Fig. 1B, we detected high molecular weight form corresponding to the glycosylated GPC3 and bands corresponding to the non-glycosylated GPC3 core protein and its cleavage product of in 3T3-L1 adipocytes as in HepG2 cells. These data were consistent with the previous reports [11,13].

Intracellular localization of GPC3

The intracellular localization of GPC3 was observed in adipocytes by laser confocal microscopy. First, we determined GPC3 protein by immunofluorescent microscopy using our specific antibody against GPC3 in HepG2 cells and primary hepatocytes as a positive or negative control, respectively (Fig. 2A). As shown in Fig. 2B, most of the GPC3 was at the plasma membrane, as reported for other cell lines [17]. As expected, we observed co-localization of GLUT4 and GPC3 after insulin stimulation (Fig. 2B). Next, in order to confirm plasma membrane integrity, we prepared plasma membrane sheets from 3T3-L1 adipocytes, stained with anti-GPC3 and anti-GLUT4 antibodies, and then observed GPC3 expression by confocal microscopy (Fig. 2C). We observed that GLUT4 translocated to the plasma membrane in response to insulin stimulation. GPC3 localized at the plasma membrane on under both conditions, and merged more clearly with GLUT4 after insulin stimulation.

The results shown in Fig. 1A and Fig. 2 suggest that GPC3 interacted with GLUT4 at the plasma membrane.

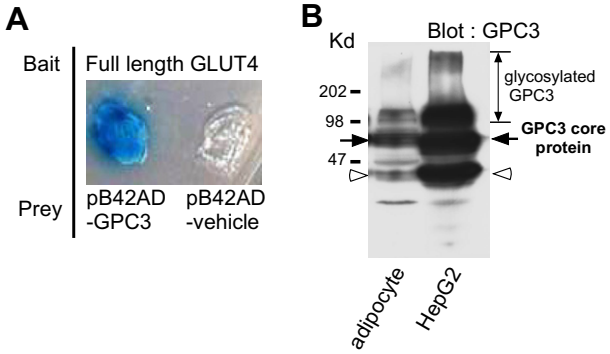

Fig. 1. Identification and expression profile of GPC3. (A) Interaction between GLUT4 and GPC3 was confirmed by the yeast two-hybrid system. Full-length cDNAs of GLUT4 and GPC3 were used as bait and prey, respectively. (B) Lysates of 3T3-L1 adipocytes and HepG2 cells were prepared, and separated by SDS–PAGE. The membrane was then blotted with anti-GPC3 antibody. HepG2 lysate was used as positive control. Arrow indicates the non-glycosylated full-length GPC3 core protein. Open arrowhead indicates a cleavage product containing the N-terminus of GPC3.