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Glypican 3 Binds to GLUT1 and Decreases Glucose Transport Activity in Hepatocellular Carcinoma Cells

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ABSTRACT

Glypican 3 (GPC3), a member of heparin sulfate proteoglycans, is attached to the cell surface by a glycosylphosphatidylinositol anchor and is reported to be overexpressed in liver cancers. In order to identify GPC3 binding proteins on the cell surface, we constructed a cDNA containing the C-terminal cell surface-attached form of GPC3 (GPC3c) in a baculoviral vector. The GPC3c bait protein was produced by expressing the construct in Sf21 insect cells and double purified using a His column and Flag immunoprecipitation. Purified GPC3c was used to uncover GPC3c-interacting proteins. Using an LC–MS/MS proteomics strategy, we identified glucose transporter 1 (GLUT1) as a novel GPC3 interacting protein from the HepG2 hepatoma cell lysates. The interaction was confirmed by immunoprecipitation (IP)–WB analysis and surface plasmon resonance (SPR). SPR result showed the interaction of GLUT1 to GPC3c with equilibrium dissociation constants (K_D) of 1.61 nM. Moreover, both incubation with GPC3c protein and transfection of Gpc3c cDNA into HepG2 cells resulted in reduced glucose uptake activity. Our results indicate that GPC3 plays a role in glucose transport by interacting with GLUT1. J. Cell. Biochem. 111: 1252–1259, 2010.

KEY WORDS: GLYPICAN 3; GLUT1; HEPATOMA; PROTEOMICS; GLUCOSE UPTAKE

Glypican-3 (GPC3) is a member of the glypican family, a group of heparin sulfate proteoglycans linked to the cell surface through a glycosylphosphatidylinositol anchor [Bernfield et al., 1999; Filmus and Selleck, 2001]. Glypican-3 regulates the signaling activity of various morphogenes, including Wnts, Hedgehogs (Hhs), bone morphogenic protein (BMP), and fibroblast growth factors (FGF) [Capurro et al., 2008]. GPC3 has been reported as a tumor biomarker in hepatomas [Nakatsura et al., 2003; Sung et al., 2003; Yamauchi et al., 2005; Wang et al., 2006]. It has also been shown that GPC3 is overexpressed in other cancers, including melanoma and breast cancers [Xiang et al., 2001; Nakatsura et al., 2004]. Full-length GPC3 proteins are about 70 kDa; however, the N-terminal 40 kDa has been shown to be cleaved and circulates in the blood [Capurro et al., 2003; Capurro and Filmus, 2005]. Thus, in this study we looked for proteins that can bind to the remaining C-terminal 30 kDa portion of GPC3, called GPC3c.

Many protein–protein interactions are part of larger cellular networks of protein–protein interactions. It has been previously reported that by interacting with IGF-2, GPC3 modulates IGF-2 activity [Pilia et al., 1996]. In addition, GPC3 is able to inhibit canonical Wnt signals involved in cell proliferation and survival. It is also able to activate non-canonical pathways [Stigliano et al., 2009]. It has recently been reported that GPC3 plays a role in glucose transport by interacting with GLUT4 [Taguchi et al., 2008]. These reports support the hypothesis that GPC3 can modulate cellular signals and metabolism by interacting with partner proteins at the cell surface.

Malignant cells require accelerated glycolysis for the generation of ATP to meet their high-energy demands for cell proliferation and survival. The facilitative glucose transporters (GLUTs) are used in creating the diffusion gradient of glucose (and other sugars) across plasma membranes and exhibit different substrate specificities, kinetic properties, and tissue expression profiles. The glucose transporter isoform 1 (GLUT1) was cloned from a HepG2 hepatoma cell line [Mueckler et al., 1985] and encodes a key rate-limiting factor in glucose transport in cancer cells. GLUT1 is expressed

Abbreviations: GPC3, glypican 3; GLUT1, glucose transporter 1; IP, immunoprecipitation; WB, Western blot.

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primarily in the brain and erythrocytes. Moderate levels of expression are also seen in adipose tissue, muscle, and liver, among others [Wood and Trayhurn, 2003]. A recent report showed that GLUT1 expression is elevated in hepatocellular carcinomas (HCCs) and promotes tumorigenesis [Amann et al., 2009]. Interestingly, one report demonstrated that GLUT1 is not expressed in normal hepatocytes [Roh et al., 2004].

Mass spectrometry (MS)-based proteomics technology development has made it possible to identify interacting proteins in a relatively simple way [Berggard et al., 2007]. In this study, we identified GLUT1 as a GPC3c-binding partner by co-IP combined with a MS-based proteomics strategy and found that GPC3c has an influence on glucose uptake activity in hepatoma cells.

MATERIALS AND METHODS

MATERIALS

An anti-FLAG M2 monoclonal antibody was purchased from Sigma (St. Louis, MO), and an anti-GLUT1 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated secondary antibody was purchased from Santa Cruz Biotechnology. ECL plus was purchased from Amersham Pharmacia Biotech (England, UK). PVDF membrane was purchased from Millipore. Purified human GLUT1 proteins were purchased from GenWay Biotech, Inc. (San Diego).

CELL CULTURE

The human HCC cell line HepG2 (ATCC, Manassas, VA) was grown in Dulbecco's modified Eagle's medium (DMEM/high glucose; Hyclone, South Logan, UT) with 10% fetal bovine serum (Hyclone) and 1% antibiotic–antimycotic (Gibco, Grand Island, NY) at 37°C.

PREPARATION OF hGPC3c RECOMBINANT PROTEINS

To obtain recombinant baculovirus, an in vitro site-specific recombination method was used. The cDNA of entry clone pEntr-hGPC3c (C-terminal portion of hGPC3, residues 1190–1856) was inserted into a vBacHTS-HisFlag baculoviral vector (Newgex, Seoul, Korea) using site-specific recombination (Fig. 1A). The first cell stock (named as passage number 0 (P0)) was obtained from Sf21 cells transfected with recombinant DNA. For small-scale protein expression, 1×10^6 Sf21 cells were seeded onto a six-well plate (SPL) and infected with 100 μl of recombinant baculovirus for 1 h, at which time 2 ml of SFM900II-SFM (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum was added. The insect cells were harvested 4 days after baculovirus infection. GPC3c proteins were isolated from insect cell lysates first using a Ni-column and then further with immunoprecipitation (IP) using anti-Flag Ab-conjugated beads.

IMMUNOPRECIPITATION AND COOMASSIE BLUE STAINING

HepG2 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 Bradford assay reagent (Bradford protein; Bio-Rad Laboratories, Hercules, CA). For IP, the cell lysates were pre-incubated with purified hGPC3c protein at 4°C for 1 h. GPC3c and the cell lysate were incubated at 4°C for 18–24 h with ANTI-FLAG M2-agarose from mouse (Sigma). After IP, eluted samples were resolved by SDS–PAGE, and the gels were stained with Bio-Safe Coomassie G250 Stain solution (Bio-Rad Laboratories) for 1 h with gentle shaking at room temperature.

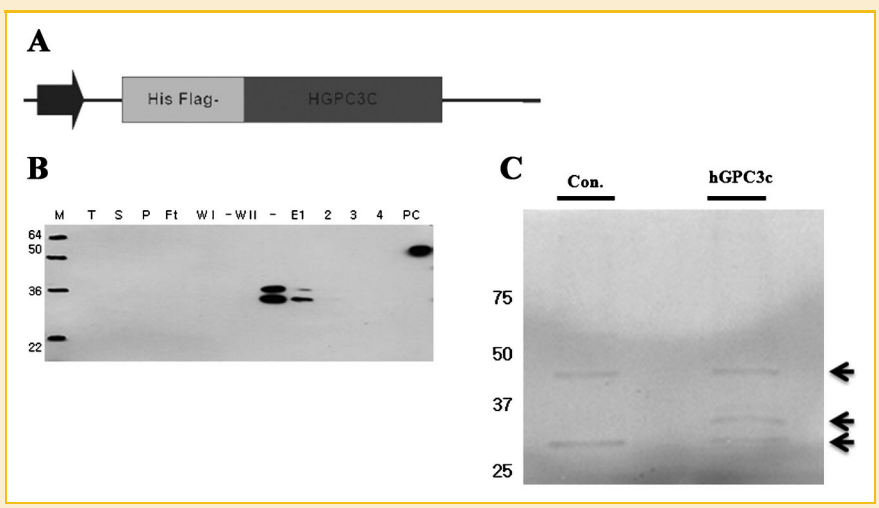

Fig. 1. Cloning and expression of hGPC3 in baculoviral vectors. A: Map of the vBacHTS-HisFlag-Gpc3c baculoviral vector. The arrow indicates the polyhedrin promoter of the vector used to express the protein in insect cells. B: Western blot analysis after Ni-NTA protein purification from the Sf21 cell of recombinant baculovirus (Newgex). Anti-HisG-HRP (1:5,000 dilution) was used to detect HisFlag-GPC3c proteins. M, Prestained protein size marker (Invitrogen); T, total lysates; S, soluble; P, precipitated; Ft, flow through (1.3 μl loaded); WI, WII, Wash I and II; E1–4, elution fraction (10 μl loading); PC, positive control HF-p53 30 ng. C: Coomassie bright blue staining after immunoprecipitation of HepG2 cell lysate and GPC3c. The middle arrow indicates the GPC3c protein after tandem purification and IP. Upper arrow: ACTG1 actin; lower arrow: GAPDH.

IN-GEL DIGESTION AND LC–ESI-MS/MS ANALYSIS

Coomassie-stained gels were in-gel digested as described previously [Heo et al., 2007]. Briefly, the gel pieces were placed in 10 μl of 25 mM ammonium bicarbonate buffer containing 20 μg/ml modified sequencing grade trypsin (Roche Applied Science, Mannheim, Germany) and incubated overnight at 37°C. The tryptic peptide mixture was eluted from the gel with 0.1% formic acid. LC–MS/MS analysis was performed using Thermo Finnigan's ProteomeX workstation LTQ linear ion trap MS (Thermo Electron, San Jose, CA) equipped with ESI sources as described previously [Heo et al., 2007].

DATA ANALYSIS

MS data were analyzed as previously described [Kim et al., 2009]. Briefly, tandem mass spectra were extracted, charge state deconvoluted, and deisotoped by Sorcerer 3.4 beta2 (Sorcerer software 3.10.4, Sorcerer Web interface 2.2.0 r334 and TransProteomic Pipeline 2.9.5). All MS/MS samples were analyzed using SEQUEST (ThermoFinnigan, San Jose, CA; version v.27, rev. 11). Sequest was set up to search the IPI Human database (IPI ver.3.29, 69965 entries) assuming that the digestion enzyme used was semi-trypsin. Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.5 Da. The iodoacetamide derivative of cysteine was specified in Sequest as a fixed modification. The oxidation of methionine, iodoacetamide derivative of cysteine were specified in Sequest as variable modifications. Scaffold (version Scaffold-01_07_00, Proteome Software, Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [Keller et al., 2002]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [Nesvizhskii et al., 2003]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

MEMBRANE PROTEIN EXTRACTION AND WESTERN BLOT

Cell membrane fractionation using the MEM-PER system was done as previously described [Qoronfleh et al., 2003] and according to the manufacturer's protocol. After SDS–PAGE, the proteins were transferred to a nitrocellulose membrane (Whatman, Germany). The membranes were incubated with anti-FLAG-M2-HRP antibody (Sigma, 1:1,000 dilution) or anti-GLUT1 antibody (Santa Cruz Biotechnology; 1:1,000 dilution) overnight at 4°C. The membrane was then washed and GLUT1 detection was performed. The primary antibodies were detected using goat anti-rabbit IgG conjugated to HRP. The bands were visualized with enhanced chemiluminescence ECL plus reagents (Amersham Pharmacia Biotech).

SPR-BASED INTERACTION STUDIES

Biomolecular interaction of GPC3c and GLUT1 were also analyzed by surface plasmon resonance (SPR) using a Biacore 2000 (Biacore, Sweden), a core facility at the Korea Basic Science Institute, Seoul. Purified GPC3c were immobilized on a CM5 biosensor chip (Biacore) as described by the manufacturer. The SPR analysis was done as previously reported [Saleem and Kim, 2009]. In brief, after chip activation with 0.4 M of *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.1 M of *N*-hydroxysuccinimide (NHS), GPC3c (in 10 mM sodium acetate buffer at pH 4.0) was passed through the flow cells. GPC3c was immobilized to a final of 2800 resonance unit (for multianalyte binding experiments), followed by capping the sensor chip with 1 M ethanolamine (pH 8.5). By injecting a series of GLUT1 (GenWay Biotech, Inc.) samples with different protein concentrations on a specified time, followed by injection of an eluent buffer (25 mM HEPES, pH 7.4, 150 mM NaCl) at 25°C. The chip surfaces were regenerated by 10 mM NaCl in 25 mM HEPES (pH 8.0). Kinetic parameters were determined using Biaevaluation 3.1 (Biacore). All binding curves were corrected for background and bulk refractive contribution by subtraction of the reference flow cells. The SPR experiments were repeated four times and similar data were acquired.

2-DG UPTAKE EXPERIMENT

The 2-DG uptake experiments were carried out as previously reported [Chirayath et al., 1998; Heo and Han, 2006]. Briefly, the 2-DG uptake was examined by removing the culture medium by aspiration and gently washing the cells twice with the uptake buffer (140 mM NaCl, 2 mM KCl, 1 mM KH_2PO_4, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 5 mM L-alanine, 5 μM indomethacin, and 10 mM HEPES/Tris, pH 7.4). After washing, the cells were incubated in uptake buffer containing 1 μCi/ml 2-DG at 37°C for 30 min. At the end of the incubation period, the cells were washed three times with ice-cold uptake buffer, and the cells were digested in 1 ml 0.1% SDS. The intracellular 2-DG uptake was determined by removing 900 μl of each sample and counting the radioactivity using a liquid scintillation counter. The remainder of each sample was used for protein determination [Bradford, 1976]. The radioactivity counts for each sample were then normalized to the protein level and corrected for the zero-time uptake per mg protein. All uptake measurements were performed in triplicate.

STATISTICAL ANALYSIS

The results are expressed as mean ± standard error (SE). All experiments were analyzed by ANOVA, followed in some cases by a comparison of the treatment and the control using the Bonnferroni–Dunn. A P-value of <0.05 was considered significant.

RESULTS

CLONING THE GPC3c CODING REGION INTO THE BACULOVIRUS VECTOR

The truncated C-terminal portion of GPC3 cDNA (residues 1190–1856, called GPC3c) was cloned into a baculovirus vector containing a His-Flag tag by PCR-mediated cloning. Figure 1A shows the construction of the vBacHTS-HisFlag-Gpc3c baculoviral vector. The cloned sequence was verified by DNA sequencing and the cloned cDNA was then expressed in Sf21 insect cells. After Ni column purification, the GPC3c protein was detected around 30 kDa (Fig. 1B).

IP–MS STRATEGY ALLOWS THE IDENTIFICATION OF GPC3c-BINDING PARTNERS

To detect proteins that interacted with GPC3c, we used a co-IP and MS strategy. To obtain pure GPC3c protein, we used tandem purification. GPC3c protein expressed in insect cells using the recombinant baculovirus vector was first purified with a Ni-column and then captured on anti-FLAG M2-agarose beads. The captured GPC3c protein was then used as bait to identify interacting proteins in HepG2 human hepatoma cells. The co-immunoprecipitated complexes were separated by SDS–PAGE and stained with Coomassie bright blue (Fig. 1C). Because the Coomassie staining has low sensitivity, we did not see any strong bands except the GPC3c bait proteins and some abundant background proteins (Fig. 1C). However, we have routinely observed many proteins in areas of the gel with no discernable Coomassie staining using LC–MS/MS analysis [Cho et al., 2009]. Each lane was cut into 15 equal regions, including the blue color bands, and each region was trypsinized and analyzed in the nano-LC–MS/MS followed by protein database searching. Tandem mass spectra were interpreted using the Sorcerer program with the SEQUEST algorithm and Scaffold program. Orthogonal filtering criteria (greater than 95.0% peptide probability and protein probability containing at least two identified peptide hits) and manual examination of the peaks were used to establish the final data.

In the biological duplicate analysis from cell culture to LC–MS/MS, after subtracting the non-specific contaminants in the control, which did not have bait GPC3c protein, a total of 11 non-redundant proteins was identified by our MS/MS analysis as interacting with GPC3c (Table I).

IDENTIFICATION OF GLUT1 AS AN INTERACTING PROTEIN WITH GPC3c

Among the 11 proteins identified by our tandem IP plus MS, we have focused on glucose transporter 1 because it is present in the membrane and is an important transporter related to cancer. A single peptide of GLUT1 protein was identified by double detection in both independent experiments (Table I). To verify that GLUT1 was identified correctly, we manually confirmed the peptide by tandem mass peaks (Fig. 2A). We then wanted to see whether the identified peptide had any homology with other glucose transporters. Alignment of the peptide sequence "TFDEIASGFR," which was identified by our MS analysis, with other members of the glucose transporter family showed that this peptide is unique to GLUT1 and does not overlap with other glucose transporters (Fig. 2B). Next, we further demonstrated the interaction of GLUT1 with GPC3c by co-IP and Western blot analysis using a GLUT1-specific antibody (Fig. 2C). Together, our results showed that the GLUT1 protein was present in a co-IP complex with GPC3c.

ANALYSIS OF GPC3c AND GLUT1 INTERACTION BY SPR

We confirmed the binding of GLUT1 to GPC3c using SPR, which is a powerful tool for real-time measurement of direct protein–protein interactions. A representative sensorgram (Fig. 3) depicts a resonance response significant of a concentration-dependent interaction between GPC3c and GLUT1. A kinetic analysis of SPR data using Biaevaluation software showed equilibrium dissociation constant (K_D) = 1.61 nM.

GPC3c DECREASED THE GLUCOSE UPTAKE IN HepG2 CELLS

Because GPC3c and GLUT1 are known to be expressed at the plasma membrane, we speculated that GPC3c might regulate glucose uptake through its interaction with GLUT1. Although it had been known that GLUT1 is a constitutive glucose transporter, it was recently reported that GLUT1 expression and its glucose uptake activity can be regulated in hepatoma cells [Amann et al., 2009]. We tested whether GPC3c has any effect on glucose uptake activity in HepG2 cells. Purified GPC3c proteins were added to HepG2 cells and glucose uptake activity relative to the dose of GPC3 was measured. To our surprise, the glucose uptake activity was significantly decreased by the addition of GPC3c into the HepG2 cell culture media (Fig. 4). Because this experiment was done by direct addition of GPC3c proteins, this may have resulted in the physical blockage of the glucose transporter at high protein concentrations. To rule out this possibility, we cloned the Gpc3c cDNA into the pcDNA3.1 mammalian expression vector and transfected HepG2 cells with the Gpc3c cDNA. The transfection experiment also showed a decrease of glucose uptake activity to a similar level (Fig. 5A). The expression of transfected Gpc3c in the total cell lysates (Fig. 5B) and in the

TABLE I. List of the Proteins Doubly Identified in Co-Immunoprecipitated GPC3c Complex by LC–MS/MS

IPI accession no.	Abbreviation	Protein description	Total peptides[a] 1st	Total peptides[a] 2nd	Coverage (%)[b]
IPI00179709	TUBA3C	Isoform 1 of tubulin alpha-2 chain	10	15	21
IPI00179330	UBA52	Ubiquitin and ribosomal protein L40 precursor	8	8	38.8
IPI00301277	HSPA1L	Heat shock 70 kDa protein 1-like variant	8	10	12
IPI00021266	RPL23A	60S ribosomal protein L23a	7	8	8.76
IPI00006482	ATP1A3	ATPase alpha 3 subunit variant	5	2	4.5
IPI00003925	PDHB	Isoform 1 of pyruvate dehydrogenase E1 component subunit beta	4	4	8.21
IPI00007084	SLC25A13	Mitochondrial aspirate-glutamate carrier protein	4	2	4.88
IPI00027251	STK38	Serine/threonine-protein kinase 38	4	2	6.24
IPI00103467	ALDH1B1	Aldehyde dehydrogenase X, mitochondrial precursor	3	10	2.71
IPI00220194	GLUT1	Facilitated glucose transporter member 1	2	2	2.3
IPI00333619	ALDH3A2	Isoform 2 of fatty aldehyde dehydrogenase	2	1	2.56

[a]Identified peptides in each duplicate experiment.
[b]Average coverage of peptides on the proteins in duplicate experiments.

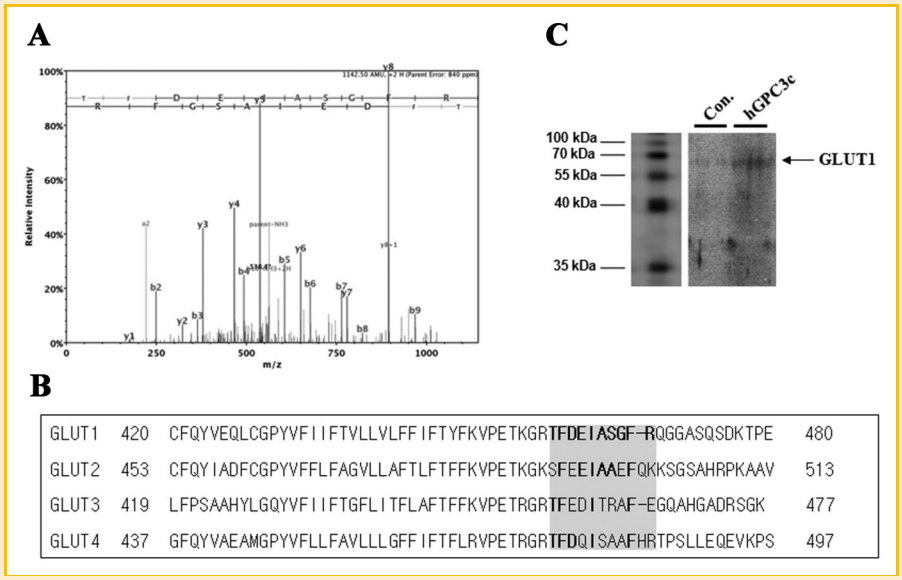

Fig. 2. Identification of GLUT1 as a binding protein to GPC3c by mass spectrometry. A: A MS/MS mass spectrum showing the peptide identification of GLUT1 protein. B: Amino acid sequence alignment of human GLUT1, GLUT2, GLUT3, and GLUT4 proteins near the TFDEIASGFR peptide identified by mass spectrometry. C: Confirmation of GLUT1 binding to GPC3c by IP and Western blot. HepG2 cells were incubated with the purified HisFlag-hGPC3c protein for immunoprecipitation. GPC3c was detected by Western blotting analysis using an anti-GLUT1 antibody.

membrane fraction (Fig. 5C) was shown by Western blot analysis. All together, these results indicate that glucose uptake activity was decreased by GPC3c.

DISCUSSION

Identification of proteins that interact with a particular target protein helps to specify the function of the protein. By co-IP and MS analysis, we have identified GLUT1 as a protein that interacts with GPC3 C-terminal protein in HepG2 hepatoma cells. Co-IP and Western blot analysis confirmed their interaction. To our surprise, GPC3c decreased glucose uptake activity in hepatoma cells after both purified GPC3c protein treatment and Gpc3c transfection.

We used the C-terminal portion of the GPC3 (GPC3c) in our study. It is known that once GPI anchored to the cell surface, the N-terminal portion of GPC3 can be cleaved and released from hepatoma cells [Nakano et al., 2009]. Our goal was to identify the function of remaining GPC3c in these cells. Therefore, we aimed to analyze the interacting proteins with the remaining GPC3c proteins.

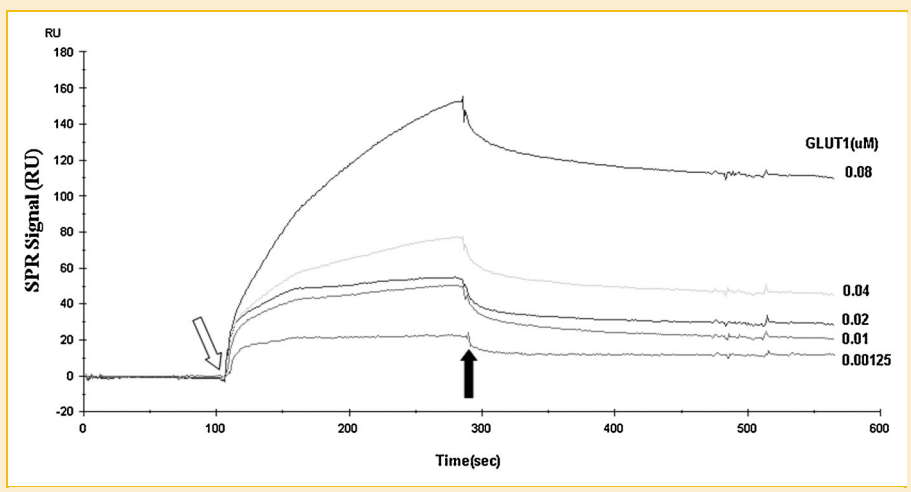

Fig. 3. SPR sensorgram of GLUT1 binding to GPC3c. One representative sensorgram demonstrating concentration-dependent binding of GLUT1 to GPC3c is shown. The white arrow indicates the start of injection of different concentrations of GLUT1 (association phase), and the black arrow indicates the start of injection of eluent buffer (dissociation phase).

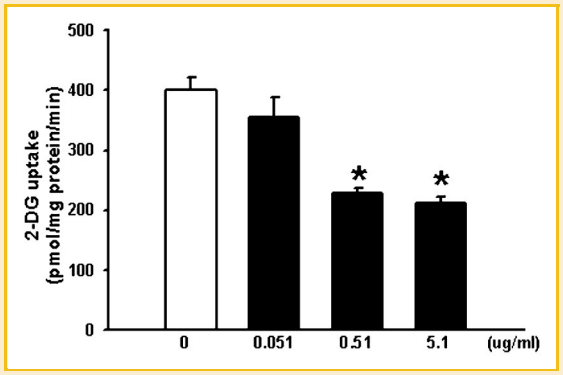

Fig. 4. Glucose uptake activity was decreased by incubation with GPC3c protein. HepG2 cells were treated with different doses of hGPC3c (0–5.1 μg/ml) for 6 h before the glucose uptake 2-DG experiment. Values shown are means ± SE of three independent experiments with triplicate dishes. $^{*}P < 0.05$ compared to control.

One of the identified GPC3c-interacting proteins was GLUT1. The ubiquitously expressed glucose transporter GLUT1 was originally known to be constitutively targeted to the plasma membrane and shows a much less dramatic translocation in response to insulin [Haney et al., 1995]. GLUT1 is located in the plasma membrane of cells throughout the body, as they are responsible for maintaining a basal rate of glucose uptake. The basal blood glucose level is approximately 5 mM. The K_m value of the GLUT1 proteins is 1 mM. Therefore, GLUT1 has a high affinity for glucose and its uptake from the bloodstream [Wood and Trayhurn, 2003]. This low-K_m GLUT1 isoform is known to be highly expressed in many cultured cancer and oncogene-transformed cells, suggesting that oncogenic transformation and some activated oncogenes may control the transcription of the GLUT1 gene [Flier et al., 1987]. Interestingly, it

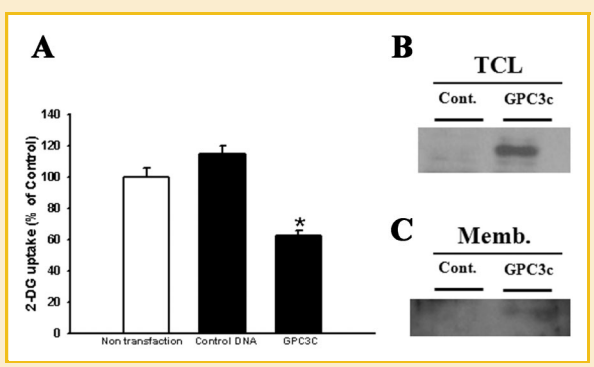

Fig. 5. Glucose uptake activity was also decreased by GPC3c gene transfection into HepG2 cells. A: hGpc3c cDNA or control vector was transfected into HepG2 cells. The glucose uptake activity was then measured as described in the Materials and Methods Section. B: hGpc3c cDNA was transfected into HepG2 cells. The cell lysates were separated by SDS–PAGE, and Western blotting was done with an anti-Flag Ab for the detection of h3PC3c. C: The membrane fraction was prepared from the cells transfected with either pcDNA3.1 (control) or hGpc3c cDNA as indicated, and the hGPC3c proteins in the membrane fraction were detected by an anti-Flag antibody.

has been reported that, upon SV 40 large-T antigen transformation, pancreatic cells expressed high levels of GLUT1 whereas it dramatically decreased GLUT2, suggesting glucose transporter isoform switch [Tal et al., 1992]. In clear cell renal cell carcinomas, GLUT1 expression increased while GLUT4 decreased [Suganuma et al., 2007].

It was also recently reported that GLUT1 is upregulated in HCC. GLUT1 gene expression was induced in HCC cells by the HIF1α transcription factor in hypoxic conditions [Amann et al., 2009]. An immunohistochemical study also showed that a tissue microarray of 152 HCC cases had a significant correlation between GLUT1 protein expression levels and a Ki-67 proliferation index, advanced tumor stages, and poor differentiation [Amann et al., 2009]. When GLUT1 was suppressed, the cell growth and migratory potential of the HCC cells were impaired, and inhibition of GLUT1 reduced both glucose uptake and lactate secretion.

The interaction of GPC3c to GLUT1 was confirmed by both IP–WB and SPR in this study. Because we observed the interaction of GPC3c with GLUT1, we speculated that the increased GPC3 protein level in hepatomas might influence GLUT1's glucose uptake function, because GLUT1 expression is also increased in hepatomas. To our surprise, the glucose uptake activity in hepatomas was decreased by GPC3c protein treatment and by Gpc3c cDNA transfection. While we were writing our manuscript, we noted that our result was in contrast to a recent publication by Taguchi et al. [2008]. In that study, overexpression of full-length GPC3 protein increased insulin-stimulated glucose uptake through interaction with cell surface GLUT4 in the cell [Taguchi et al., 2008]. Both GLUT1 and GLUT4 are 12-pass transmembrane proteins (12TM) whose carboxyl termini may dictate their cellular localization [Haney et al., 1995].

This difference can be explained by several possibilities that need to be tested in future studies. First, it might be due to differences in the protein used in experiments. That is, we used the C-terminal portion of the GPC3 proteins (GPC3c) as bait, whereas Taguchi et al. used full-length GPC3. Thus, it can be imagined that in a certain stage (possibly the fast growing phase) of hepatoma development, the increased GPC3 protein binds to GLUT4 and helps to increase glucose uptake activity. However, in the hypoxic condition of the hepatoma cells in the center of tumors, where GLUT1 levels are increased, the oxygen and glucose supplies are limited and GPC3 proteins are cleaved. The remaining C-terminal GPC3c can bind to the GLUT1 and reduce the glucose uptake activity. However, to support our hypothesis, we first need to test whether the N-terminal cleavage of GPC3 is increased in the hypoxic condition of hepatomas. In a future study, the domains of GPC3c and GLUT1 that bind to one another need to be clarified to show the exact regulatory mechanisms of GPC3c's role in the decrease of glucose uptake activity in hepatoma cells.

There have been some reports showing that GPC3 might work as an oncofetal protein in carcinogenesis by interacting with insulin-like growth factor-signaling pathway [Wang et al., 2006; Cheng et al., 2008]. The reduction of glucose uptake activity by GPC3c is supported by the indication that GPC3 may play a role as a metastasis suppressor during cancer. GPC3 is also able to inhibit canonical Wnt signaling, which is involved in cell proliferation and

survival. It has been reported that the malignant properties of breast tumor cells can be reverted, at least in part, by GPC3 modulation of Wnt signaling, suggesting the potential role of GPC3 as a metastasis suppressor [Stigliano et al., 2009].

In conclusion, we identified the novel GPC3c-binding protein GLUT1 using MS-based proteomics analysis. Our data showed that GPC3c decreased glucose uptake activity in the hepatoma HepG2 cells. GPC3c-binding GLUT1 may play a role in glucose metabolism.

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