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Differential localization of glucose transporter isoforms in non-polarized mammalian cells: distribution of GLUT1 but not GLUT3 to detergent-resistant membrane domains

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Abstract

The hexose transporter family, which mediates a facilitated uptake in mammalian cells, consists of more than 10 members containing 12 membrane-spanning segments with a single N-glycosylation site. However, it remains unknown how these isoforms are functionally organized in the membrane domains. In this report, we describe a differential distribution of the glucose transporter isoforms GLUT1 and GLUT3 to detergent-resistant membrane domains (DRMs) in non-polarized mammalian cells. Whereas more than 80% of cellular proteins containing GLUT3 in HeLa cell lines was solubilized by a non-ionic detergent (either Triton X-100 or Lubrol WX) at 4 °C, GLUT1 remained insoluble together with the DRM-associated proteins, such as caveolin-1 and intestinal alkaline phosphatase (IAP). These DRM-associated proteins and the ganglioside GM1 were shown to float to the upper fractions when Triton X-100-solubilized cell extracts were centrifuged on a density gradient. In contrast, GLUT3 as well as most soluble proteins remained in the lower layers. Furthermore, perturbations of DRMs due to depletion of cholesterol by methyl-β-cyclodextrin (mβCD) rendered GLUT1 soluble in Triton X-100. Immunostaining patterns for these isoforms detected by confocal laser scanning microscopy in a living cell were also distinctive. These results suggest that in non-polarized mammalian cells, GLUT1 can be organized into a raft-like DRM domain but GLUT3 may distribute to fluid membrane domains. This differential distribution may occur irrespective of the N-glycosylation state or cell type.

Keywords: Mammalian glucose transporter; GLUT1; GLUT3; Detergent-resistant membrane; Cholesterol; N-glycosylation; HeLa cell

1. Introduction

Hexose transporters represent a family of intrinsic membrane proteins that mediate a facilitative uptake of hexose by mammalian cells [1]. Previously, five functional isoforms designated GLUT1–GLUT5 have been characterized [1] and additional novel genes that encode hexose transporter-like proteins GLUT6–GLUT11 have also been identified [2]. Although the functional characterization of these new members has not been completed, the members differ in terms of tissue distribution, transport kinetics, and substrate specificity. GLUT1 is distributed in many cells and its increased expression is one of the most characteristic changes associated with cell growth and malignant transformation [3–6]. GLUT3 is also expressed in many tissues, including brain, placenta and kidney [7], as well as in several human tumors [8,9]. In contrast, GLUT2 and GLUT4 as well as some novel glucose transporters (GLUTs) show a tissue-specific distribution (GLUT2, liver and pancreas; GLUT4, muscle and fat; GLUT8, blastocysts; GLUT9, brain and leucocytes; GLUT11, heart and skeletal muscle; etc.). GLUT5, which is expressed at highest levels in the small intestine, was found to be a fructose transporter [1]. Despite the high degree of sequence homology, each isoform can be subject to distinct modes of regulation for cellular localization and transport activity. For instance,

Abbreviations: GLUTs, glucose transporters; DRM, detergent-resistant membranes; IAP, intestinal alkaline phosphatase; GPI, glycosylphosphatidyl-inositol; TGN, the trans-Golgi network; CGL1, non-tumorigenic HeLa cell hybrid; CGL4, tumorigenic HeLa cell hybrid; CHO-K1, Chinese hamster ovary cell; ECL, enhanced chemiluminescence kit; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; CBB, Coomassie brilliant blue; mβCD, methyl-β-cyclodextrin; HLB, hydrophilic–lipophilic balance; GM_1, a ganglioside GM_1; CTXB, cholera toxin B subunit

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GLUT1–3, GLUT5 and GLUT9 localize at the cell surface, whereas GLUT4 or GLUT8 exhibits insulin-sensitive translocation from intracellular vesicles to the cell surface [1,2,10].

We have recently investigated tumor-associated alterations in glucose transporter expression in human cell hybrids derived from cervical carcinoma HeLa cells and normal fibroblasts [11–13], whose tumorigenicity may be controlled by a putative tumor suppressor gene on chromosome 11 [14]. In these studies, we found that the tumor-suppressed hybrid cells express GLUT1 alone, whereas tumorigenic cell hybrids express both GLUT1 and GLUT3 as larger forms, probably due to modifications of N-glycosylation [11–13]. However, differences in the membrane distribution and roles of these isoforms remain largely unknown.

GLUT1 and GLUT3 share many similarities in structure and function; they have 12 membrane-spanning segments with a single N-linked oligosaccharide chain on the outer surface. About 65% of their amino acid sequence is homologous, but their C-terminal domains and the extracellular loops are distinctive [1,7]. These isoforms have a high affinity to D-glucose when expressed at the cell surface [15,16]. A striking difference between them is reported in cellular localization; in polarized epithelial cells, such as Caco-2 and MDCK cells, GLUT1 is expressed on the basolateral surface, while GLUT3 is sorted to the apical surface [17,18]. In platelets and neuronal cells, GLUT3 is also present in intracellular vesicles [19,20]. These results imply distinctive roles for these transporters in mammalian cells.

Recent studies suggested that the plasma membrane in mammalian cells is not homogeneously organized, containing specific microdomains, known as detergent-resistant membranes (DRMs), lipid rafts or caveolae [21–26]. These microdomains are enriched with cholesterol and sphingolipids to organize an ordered lipid phase, including some proteins such as caveolin, glycosylphosphatidyl-inositol (GPI)-anchored proteins and Src-kinases. Mainly due to their ordered lipid nature, these membrane domains are relatively resistant to solubilization by non-ionic detergents. These DRM domains are also functionally distinguishable, being involved in various cellular events, such as signal transduction and membrane trafficking [21–26]. The presence of these microdomains under physiological conditions has been shown with several procedures [26,27] and selective pathways for sorting proteins through the trans-Golgi network (TGN) to DRM domains or the polarized cell surface are proposed in various cells [25]. In tumorigenic HeLa cell hybrids, expression of caveolin-1 and intestinal alkaline phosphatase (IAP), which may be components of the DRM, was also modulated [28,29].

In the present study, we examined if there is any differential distribution of the glucose transporter isoforms in regard to DRM domains in these cells. Here, we report for the first time that GLUT1 distributes to a cholesterol-rich DRM domain, whereas GLUT3 localizes in a fluid membrane domain. The differential distribution of GLUT1 and GLUT3 may occur irrespective of the N-glycosylation state, the tumorigenic state or cell type, implying that it is mainly due to intrinsic properties.

2. Materials and methods

2.1. Chemicals

Rabbit polyclonal antibody against C-terminal peptides of human GLUT1 and GLUT3 were purchased from Chem-

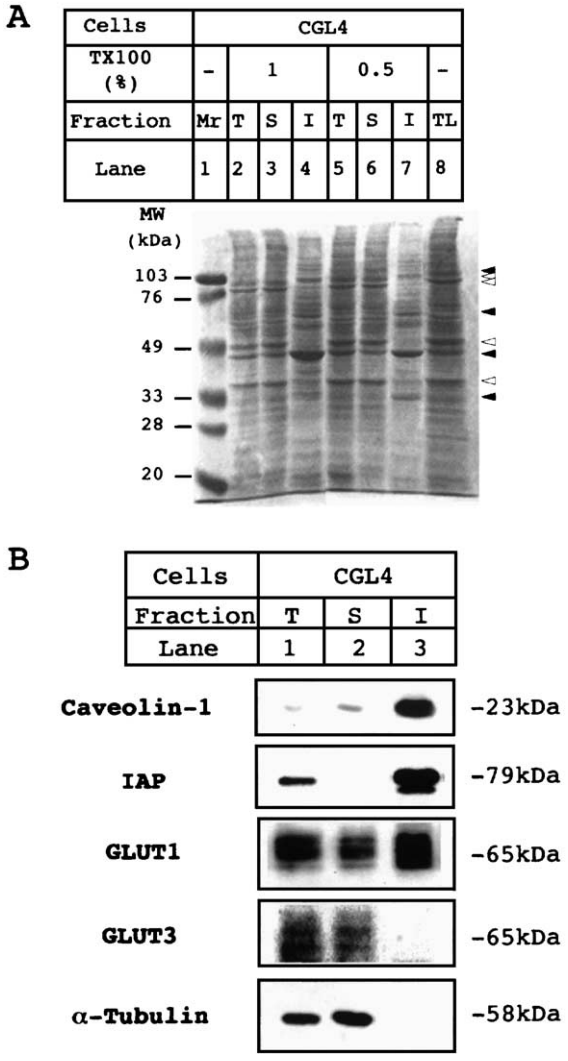

Fig. 1. Detergent-solubility of ceveolin-1, IAP, GLUT1 and GLUT3 in human cell hybrids. HeLa cell hybrids, CGL4 cells, were solubilized by either 1% or 0.5% Triton X-100 at 4 °C. Total cell lysate (T) was separated into soluble (S) and insoluble (I) fractions by centrifugation at 12,000 × *g* for 30 min, and each sample (10 μg protein/lane) was subjected to SDS-PAGE, followed by Coomassie blue staining (A). The arrows indicate proteins which were enriched (closed arrows) or reduced (open arrows) in the insoluble fraction. (B) CGL4 cells were solubilized by 0.5% Triton X-100, and subsequently fractionated samples (10 μg protein/lane of T, S, and I), prepared in (A), were similarly examined by SDS-PAGE and immunoblotting for caveolin-1, IAP, GLUT1, GLUT3 and α-tubulin. The corresponding molecular masses are indicated in kDa.

icon (CA). Affinity-purified anti-GLUT3 rabbit polyclonal antibody against C-terminal peptide was obtained from MBL, Tokyo. Polyclonal antibodies against bovine IAP (CALZYME, CA), human caveolin-1 (BD Transduction Lab, KY), human transferrin receptor (CD71; Santa Cruz, CA), and human clathrin HC (Santa Cruz) were used. Mouse monoclonal antibody against α – tubulin was provided by Sigma (MO). An enhanced chemiluminescence kit (ECL) and [1α, 2α (*n*)-^{3}H]cholesterol (1 mCi/ml) were obtained from Amersham Pharmacia Biotech (Tokyo).

2.2. Cell culture

The human cell hybrids, CGL4 (tumorigenic) and CGL1 (non-tumorigenic), are subclonal derivatives of a parental tumor-suppressed cell hybrid of HeLa D98/AH2 cells and normal human fibroblasts [11]. These cell hybrids and a human cervical carcinoma cell line, HeLa-S3, were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL) containing 5% fetal calf serum (FCS, MBL), penicillin (100 U/ml) and streptomycin (100 μg/ml) under humidified 5% CO_2/95% air at 37 °C, as described previously [11]. The GLUT3-transfectant which stably overexpressed GLUT3 in CGL4 cells (CGL4/Glut3) and CGL1 cells (CGL1/Glut3) were maintained in DMEM containing G418 (600 μg/ml), 5% FCS, penicillin and streptomycin under 5% CO_2 at 37 °C as described previously [13]. The rabbit GLUT1 cDNA [16] in an expression vector (pME18 Sneo) was similarly transfected into CGL4 cells and a stable clone, CGL4/Glut1, which overexpressed GLUT1 was isolated and determined. These cells were free from mycoplasma contamination.

2.3. Cell lysate and immunoblotting

Cell lysates in lysis buffer (150 mM NaCl, Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and 0.5% NP-40) were prepared, as described previously [13], and the

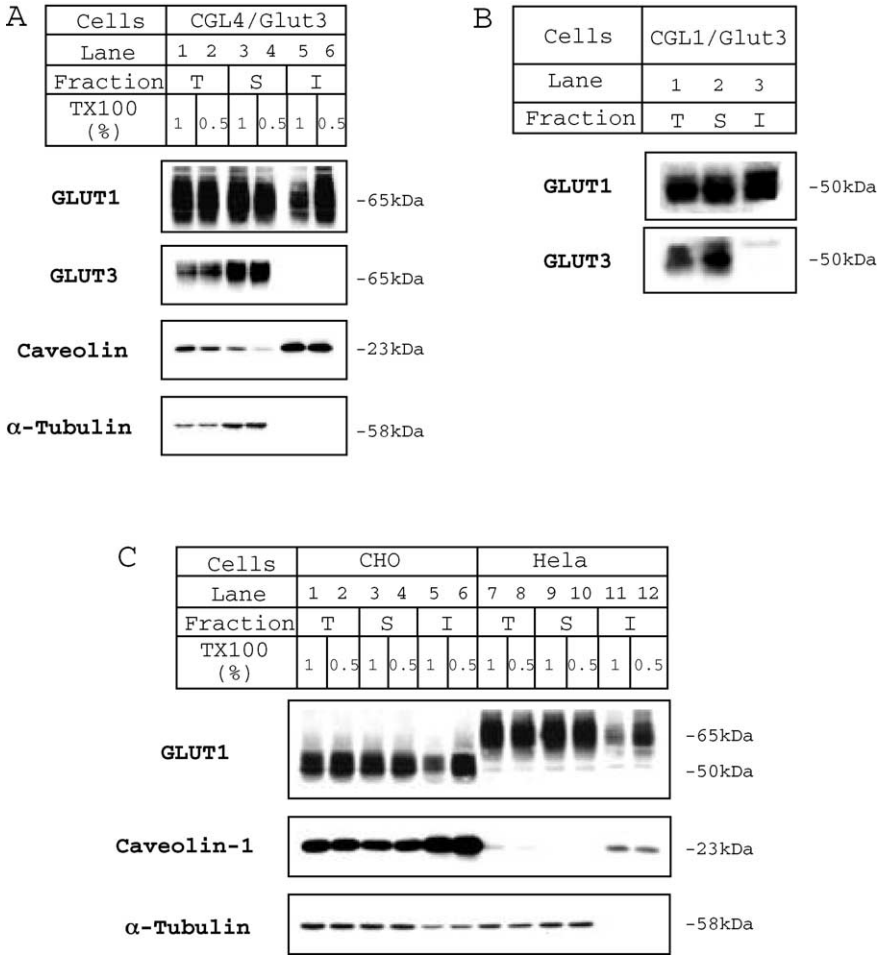

Fig. 2. Detergent-insolubility of GLUT1 in cDNA-transfected HeLa cell hybrids or in different cell types. (A) Stable transfectants of CGL4 cells, which overproduced GLUT3 (CGL4/Glut3), were solubilized by Triton X-100 (1% or 0.5%) at 4 °C, and fractionation and immunoblotting for GLUT1 and GLUT3 were performed, as described in Fig. 1. (B) A similar experiment was performed with CGL1/Glut3, which overproduce GLUT3 in non-tumorigenic CGL1 cells. (C) The DRM-distribution of GLUT1 in different cell types was also examined in HeLa-S3 cells and CHO-K1 cells solubilized by Triton X-100 (0.5% or 1%) at 4 °C. Fractionation and immunoblotting for GLUT1, caveolin-1 and α-tubulin were performed, as described in Fig. 1.

protein concentration was determined using BCA reagent (Pierce) with bovine serum albumin (BSA) as a standard. Protein samples (5–10 μg) were subjected to SDS-10% PAGE, and transferred to immobilon-P membranes (Millipore), which were incubated in TBS-Tween (500 mM NaCl, 20 mM Tris–HCl, pH 7.5, plus 0.1% Tween 20) containing 5% skim milk (Difco), followed by rabbit polyclonal antibody or mouse monoclonal antibody (1:1000–2000). The membranes were further incubated with HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Amersham Pharmacia Biotech), and visualized with the ECL-detection kit.

2.4. Detergent solubilization

The cells growing in two 10-cm dishes (HeLa-S3 and HeLa cell hybrids, about 1×10^7 cells/dish; CHO-K1, about 5×10^6 cells/dish) were washed twice with cold PBS, and treated with 0.5 ml of Hepes buffer containing 1–0.5% Triton X-100 (Sigma), 10 mM sodium Hepes, 150 mM NaCl, 5 mM EDTA and 0.5 mM PMSF for 30 min at 4 °C with gentle shaking. Alternatively, the cells which had been washed and collected in a 1.5 ml tube were similarly solubilized by Triton X-100 at 4 °C. The treated cells (about $1–2 \times 10^7$ cells) were mixed and an aliquot was kept as the total fraction (T). The remainder were centrifuged at 13,000 rpm (12,000 × *g*) for 30 min at 4 °C and the supernatant was used as the soluble fraction (S). The pellet was washed in 1 ml of cold Hepes buffer without detergent and was centrifuged at 13,000 rpm for 10 min at 4 °C. This washing procedure was repeated. The pellet (insoluble fraction, I) was solubilized with 0.2–0.5 ml of lysis buffer. A similar solubilization was performed with non-ionic detergent Lubrol WX (Lubrol 17A17; Serva). The protein concentrations were determined as described above. As a control experiment, solubilization at 37 °C instead of 4 °C was performed.

2.5. Flotation assays

Detergent-insoluble complexes were analyzed by a flotation method with Nycodenz, as described previously [30]. Briefly, confluent CGL4 cells growing in two 10-cm dishes (about 2×10^7 cells) were collected in a tube and incubated with 450 μl of MN buffer (25 mM MES, pH 6.5, and 150 mM NaCl) supplemented with protease inhibitors (GIBCO-BRL) and either 1% or 0.5% TX-100 for 15 min at 4 °C. Whole lysate was then adjusted to 35% with ice-cold 70% Nycodenz prepared in MN buffer and loaded at the bottom of ultracentrifuge tubes (Beckman TLS-55). An 8–35% Nycodenz linear step gradient in MN buffer was overlaid on the lysate (200 μl each of 25%, 22.5%, 20%, 18%, 15%, 12%, and 8% Nycodenz in MN buffer) and tubes were spun at 55,000 rpm (200,000 × *g*) for 4 h at 4 °C in a swing rotor. Twelve fractions of 180 μl each were collected from the top of the tube, from which 25 μl each was reserved for analyses on protein determination and cholera-toxin binding. Proteins in each fraction were then precipitated with 1 ml of methanol and dissolved in either 50 μl (fractions 1–10) or 100 μl (fractions 11–12) of 2 × sample buffer, subjected to SDS-PAGE analysis and immunoblotted with 5 μl of each fraction.

2.6. Detection of GM1 by cholera-toxin binding

As described in Ref. [30], 5 μl of whole lysate or each fractionated sample was diluted with 0.5 ml of MN buffer and 50 μl of each diluent was spotted onto an immobilon-P membrane. After being washed with MN buffer, the membrane was pre-incubated in 5% blocking solution (Blockace, Dainippon Pharm. Co., Osaka, Japan) for 1 h at room temperature, before being incubated with HRP-conjugated cholera toxin B subunit (CTXB-HRP) at 1:1000 in TBS-Tween containing 50% Blockace in MN buffer for 1 h. The membrane was washed three times with TBS-Tween, and visualization achieved with the ECL-detection kit, as described above.

2.7. Depletion of membrane cholesterol

CGL4 cells grown in 3 cm dishes were pre-labeled with [^{3}H]cholesterol (0.25 μCi/ml) in 0.5% FCS-containing DMEM for 20 h at 37 °C in 5% CO_2, as described by Fukasawa et al. [31]. After washing the cells with PBS, the labeled cells were incubated for 30 min at 37 °C in 5% CO_2 with 2 ml of serum-free DMEM in the presence of the indicated concentrations of methyl-β-cyclodextrin (mβCD) (Sigma). The amount of [^{3}H]cholesterol incorporated into the treated cells was determined by scintillation counting after extraction by the method of Bligh and Dyer [32], and is

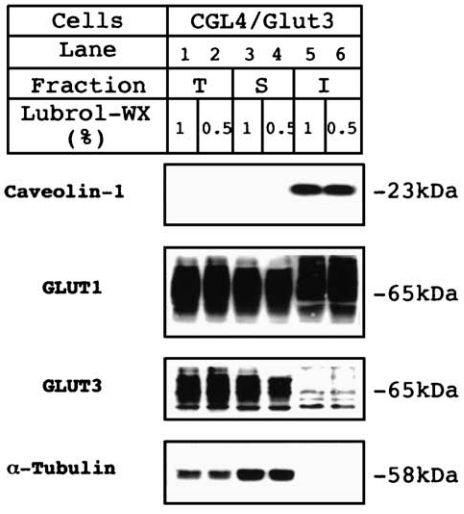

Fig. 3. Solubilization of membrane proteins by Lubrol WX. CGL4/Glut3 cells were solubilized by 1% or 0.5% non-ionic detergent Lubrol WX at 4 °C. Total cell lysate (T) was fractionated into soluble (S) and insoluble (I) fractions, and each sample (10 μg protein/lane) was subjected to SDS-PAGE and immunoblotting, as indicated.

expressed in cpm/dish. A similar cholesterol-depletion by mβCD was performed with CGL4 cells or CGL4/Glut3 cells grown in 10 cm dishes, and solubilization by Triton X-100, followed by fractionation and immunoblotting as described above.

2.8. Immunofluorescence analysis by confocal microscopy

CGL4/Glut3 cells were grown on glass cover-slips. Cells were fixed and permeabilized with 3.7% formaldehyde in PBS containing 0.2% Triton X-100 for 10 min at room

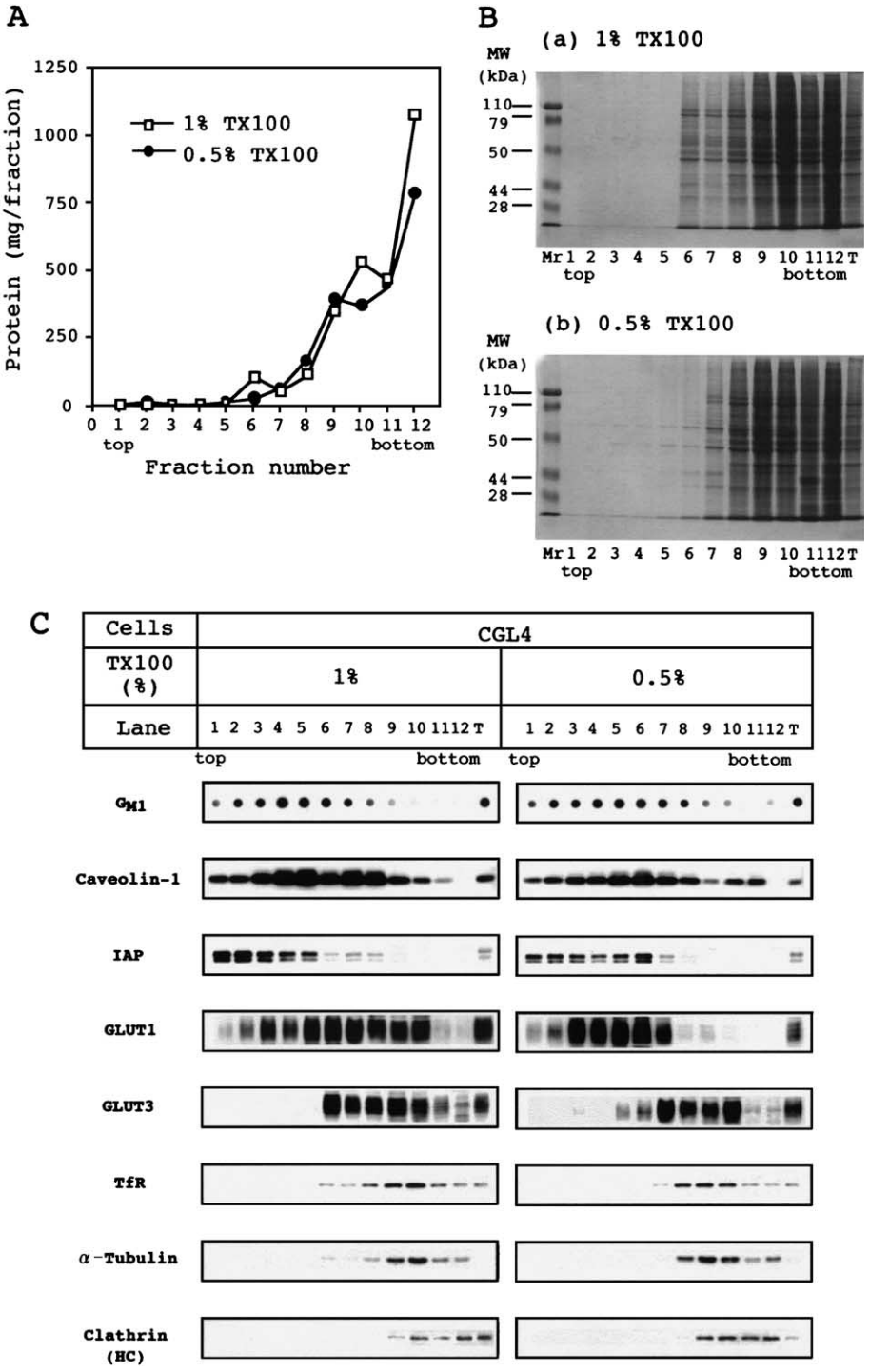

Fig. 4. Flotation assay for solubilized proteins. CGL4 cells were solubilized by 0.5% or 1% Triton X-100 at 4 °C and total cell lysate (T) was subjected to Nycodenz density gradient centrifugation. Each tube was fractionated into 12 samples, and an aliquot of each fraction was subjected to protein determination (A) or SDS-PAGE analysis and staining (B). In (C), each diluent was blotted onto a nylon membrane and the distribution of GM1 was determined from the binding of CTXB. In a separate experiment, an aliquot as determined in (B), was separated by SDS-PAGE, and immunoblottings for the indicated proteins were performed. A single blotting membrane was used for the repeated probing. One result representative of at least three different determinations is shown here.

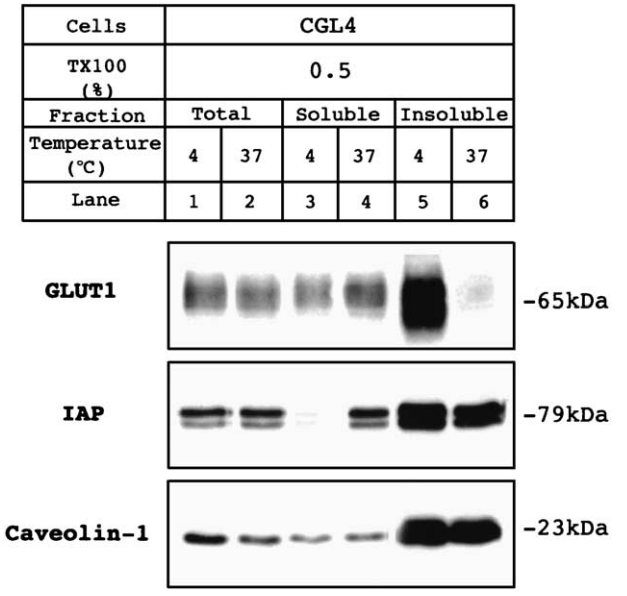

Fig. 5. Effect of increased temperature on detergent solubilization. CGL4 cells were solubilized by 0.5% Triton X-100 for 30 min at either 4 °C or 37 °C. Total cell lysate (T) was similarly fractionated into soluble (S) and insoluble (I) fractions, and each sample (10 μg protein/lane) was subjected to SDS-PAGE and immunoblotting, as described in Fig. 1.

temperature. After the blocking of non-specific binding sites with staining buffer (PBS containing 0.1% BSA) for 10 min, samples were treated with anti-GLUT1 or anti-GLUT3 together with anti-Annexin II antibody (BD Transduction Lab) in staining buffer for 1 h, followed by incubation with Alexa 488 or Alexa 594 secondary antibodies (Molecular Probes) for 1 h at room temperature. The cells were washed three times with PBS and mounted on glass slides with 80% glycerol. In all cases, cells were visualized by confocal laser scanning microscopy (Zeiss LSM 510). For the evaluation of two-color experiments, digital images were overlaid electronically and processed with PHOTOSHOP (Adobe).

3. Results

3.1. Differential solubilization of GLUT1 and GLUT3 by Triton X-100

To compare the solubility of GLUT1 and GLUT3 in non-ionic detergents, tumorigenic HeLa-derived hybrid cells, CGL4, were treated with either 0.5% or 1% Triton X-100 at 4 °C, and the solubilized (S) and insoluble (I) fractions were separated by centrifugation at 12,000 × *g*. This procedure yielded more than 80% of total protein (T) as the soluble fraction, which was stained by Coomassie brilliant blue (CBB), as shown in Fig. 1A. As little as 10% of total protein was isolated as the insoluble fraction, whose Coomassie-staining pattern was distinctive from that of the soluble fraction, as indicated by several arrows. These CBB-staining profiles of the detergent-insoluble fractions were nearly identical when the cells were solubilized by either 1% or 0.5% Triton X-100.

Immunoblotting of these samples indicated that IAP, which is expressed in CGL4 cells but not in non-tumorigenic CGL1 cells [28,29], was exclusively present in the 0.5% Triton X-100-insoluble fraction (Fig. 1B). Similarly, caveolin-1, which is a major component of the caveolae structure and well-known as a detergent-insoluble component [21–26], was also abundant in this insoluble fraction, although expression of caveolin-1 was reduced in CGL4

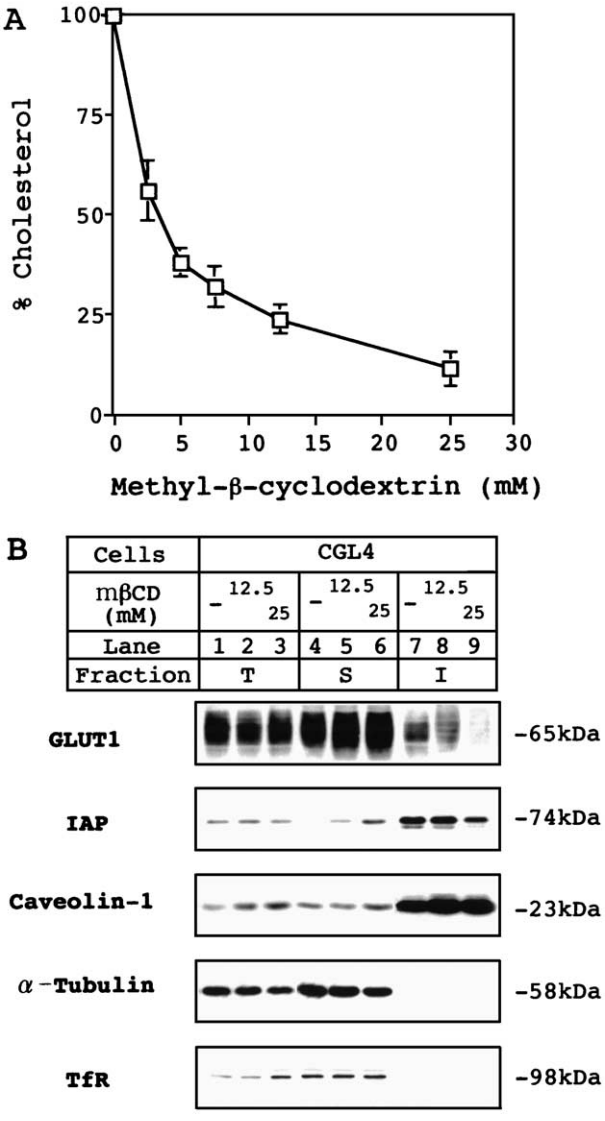

Fig. 6. Effect of cholesterol depletion on solubilization by Triton X-100. (A) CGL4 cells, which had been pre-labeled with [^{3}H]cholesterol, were treated with the indicated concentrations of mβCD at 37 °C for 30 min, and changes in [^{3}H]cholesterol were analyzed. After a similar treatment with mβCD, CGL4 cells (B) or CGL4/Glut3 cells (C) were solubilized by Triton X-100 (0.5% or 1%) at 4 °C, and the fractionation and immunoblotting of each fraction were performed, as indicated. In (D), 25 mM mβCD-treated or untreated CGL4 cells were solubilized by 0.5% Triton X-100 at 4 °C, the cell lysate was subjected to Nycodenz density gradient centrifugation, and the distribution of GM1 and immunoblotting for GLUT1 and caveolin-1 were determined.

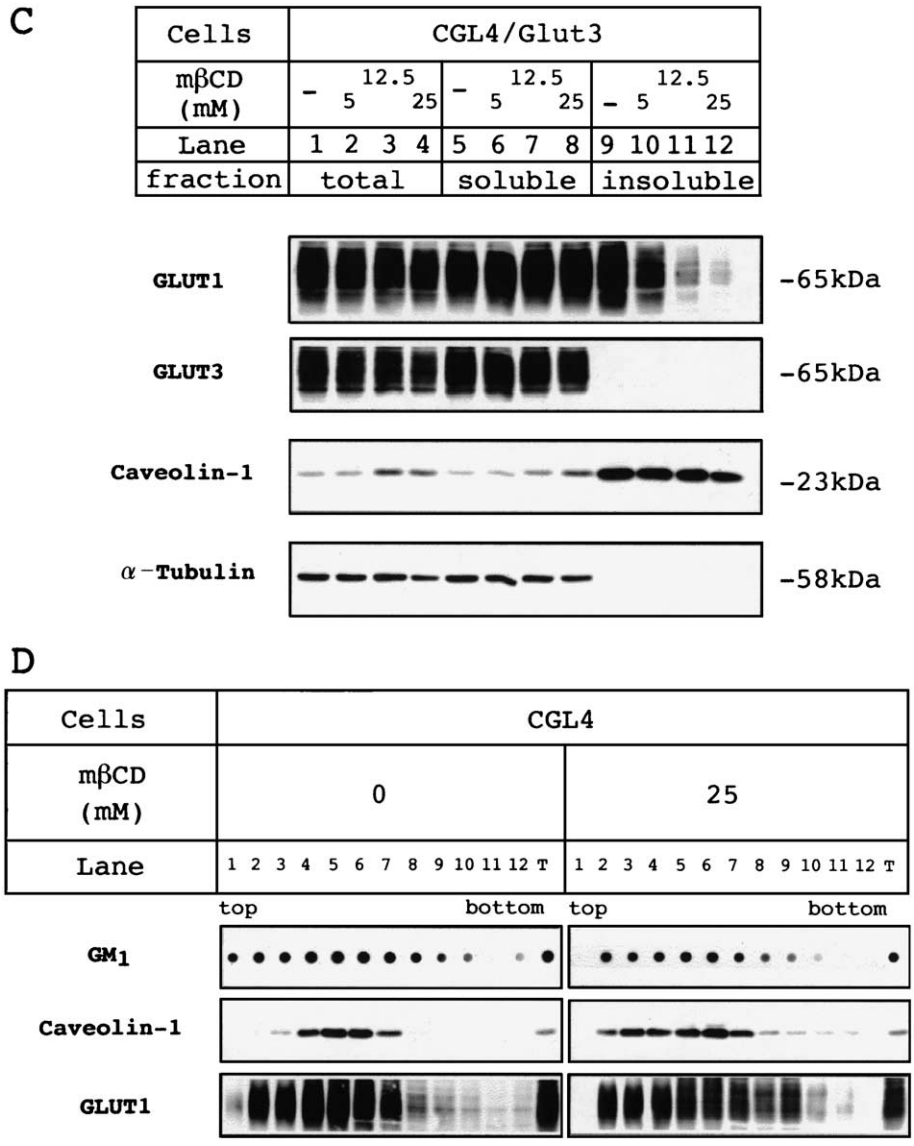

Fig. 6 (*continued*).

cells compared to tumor-suppressed CGL1 cells [29]. Next, we examined the distribution of GLUT1 and GLUT3. GLUT3 was fully solubilized by 0.5% Triton X-100 at 4 °C (Fig. 1B). In contrast, GLUT1 remained in the insoluble fraction, although it was also distributed in the soluble fraction.

3.2. Differential solubility of GLUT1 and GLUT3 in non-ionic detergents in transfectants which overproduce GLUT3 or GLUT1

To examine whether the difference in the distribution of these isoforms to the detergent-soluble or insoluble fraction is due to quantitative differences in the cells, a similar solubilization procedure was performed with transfectants which stably overproduced either GLUT3 or GLUT1 in HeLa cell hybrids. In GLUT3-transfected CGL4 cells, CGL4/Glut3, the overproduced GLUT3 was entirely solubilized by 0.5% Triton X-100 at 4 °C without an effect on the distribution of GLUT1, caveolin-1 and IAP in the insoluble fraction (Fig. 2A). Similar results were obtained with solubilization by 1% Triton X-100, although the proportion of GLUT1 associated with DRM was reduced. The overexpressed GLUT1 in CGL4 cells was similarly distributed to both fractions, as seen with parental CGL4 cells (data not shown). In any case, α-tubulin was fully solubilized under these conditions, as a positive marker for solubilization.

The differential solubility of GLUT1 and GLUT3 was further examined in different types of cells. Since a tumor-suppressed HeLa cell hybrid, CGL1, expresses GLUT1 alone, an experiment was performed with GLUT3-transfected CGL1 cells (CGL1/Glut3). In this transfectant, GLUT1 was similarly retained in the insoluble fraction

when it was treated with 0.5% Triton X-100 at 4 °C, while the overproduced GLUT3 was fully solubilized by this detergent (Fig. 2B). Thus, the differential distribution of GLUT1 and GLUT3 to DRMs seems to be independent of a tumorigenic or N-glycosylated state, since N-glycosylation of both GLUT1 and GLUT3 in tumorigenic CGL4 cells was modulated to larger forms [11–13].

A similar resistance of GLUT1 to cold Triton X-100 was observed in HeLa-S3 cells and Chinese hamster ovary (CHO-K1) cells (Fig. 2C), in which α-tubulin was fully solubilized, although the highly expressed caveolin-1 in CHO cells was partly solubilized. The insoluble nature of GLUT1 but not GLUT3 was also evident when CGL4/Glut3 cells were treated with another non-ionic detergent, Lubrol WX, whose hydrophilic–lipophilic balance (HLB) is greater than that of Triton X-100 (Fig. 3). In this experiment, α-tubulin was fully solubilized, while caveolin-1 was retained in the insoluble fraction.

3.3. Flotation profiles of solubilized membrane proteins

To examine the detergent-resistant nature of GLUT1 based on its distribution to a raft-like lipid domain, whole cell lysates solubilized by Triton X-100 at 4 °C were centrifuged on a density gradient. By this procedure, fully solubilized proteins as well as cytosolic proteins remain in the bottom layer in which the sample is loaded, whereas insoluble proteins in lipid-rich vesicles or DRM domains float on the upper layer [23–26].

Whole CGL4 cell lysates, solubilized by either 0.5% or 1% Triton X-100 at 4 °C, were centrifuged with a step gradient of 5–35% Nycodenz, and fractionated to 12 tubes. As shown in Fig. 4A, most soluble proteins were recovered from fractions 8 to 12. Consistent with this, proteins in these fractions were heavily stained with CBB after separation by SDS-PAGE (Fig. 4B). Although few proteins were detected by CBB-staining in the lower density fractions, a flotation marker for the lipid-rich vesicles, ganglioside GM1, which was detected by CTXB-binding, broadly floated to the upper fractions between 2 and 6 in both samples (Fig. 4C). A DRM-associated protein, IAP, also floated up to these fractions, around 2 to 6. In addition, Caveolin-1 floated in the upper fractions, but was distributed more widely than IAP. Most GLUT1 was co-distributed with IAP in the upper fractions when cells were solubilized by 0.5% Triton X-100, but it was more widely distributed to the lower fractions when solubilized by 1% Triton X-100. In contrast, GLUT3 was mostly distributed in the bottom layers, between 7 and 10, containing the transferrin receptor (TfR), clathrin heavy chain (HC) and α-tubulin. The differential flotation profiles between GLUT1 and GLUT3 were reproduced in transfectants which overproduce either GLUT3 or GLUT1 (data not shown). These flotation assays further support that GLUT1 but not GLUT3 distributes to the DRM or raft-like lipid domains.

3.4. Increased temperature of solubilization

In all of the experiments described above, solubilization was performed at 4 °C. Since phase partitioning by lipids shows a strong temperature dependence [21], we also solubilized membranes at 37 °C to increase stringency. The disappearance of GLUT1 from the Triton X-100-insoluble fraction was evident on solubilization of CGL4 cells at 37 °C (Fig. 5). A small amount of IAP was also solubilized at 37 °C, but most IAP remained in the insoluble fraction. However, the solubility of caveolin-1 in Triton X-100 was unaffected by the increased temperature, suggesting that among these membrane proteins, caveolin-1 is most tightly associated with the DRM domain(s) in CGL4 cells.

3.5. Effect of cholesterol depletion on solubilization

Since one of the major components of the detergent-resistant lipid domains, DRM or rafts, is cholesterol [21–24], it would be important to examine whether depletion of cholesterol affects solubilization of DRM-associated GLUT1 by Triton X-100. For this purpose, the depletion of cholesterol by mβCD was monitored by the decrease in the amount of [^{3}H]cholesterol from the labeled cells, as described by Fukasawa et al. [31]. A marked depletion of [^{3}H]cholesterol was induced by mβCD during incubation with CGL4 cells for 30 min at 37 °C in a dose-dependent manner, and more than 60% of total cholesterol was depleted by 10–25 mM mβCD (Fig. 6A). Accordingly, GLUT1, which had remained in the insoluble fraction in 0.5% Triton X-100, was rendered soluble in the cholesterol-depleted cells (Fig. 6B). In contrast, even though cholesterol depletion by mβCD was maximal, the IAP solubilized by Triton X-100 was minimal, and the insolubility of caveolin-1 was nearly unaffected. The increased solubility of GLUT1 in cold Triton X-100, which was dependent upon cholesterol depletion, was similarly observed in CGL4/Glut3 cells, in which solubilization of GLUT3 as well as α-tubulin was unaffected by the treatment with mβCD (Fig. 6C), indicating that the non-ionic detergent-resistant nature of GLUT1 is reduced by cholesterol depletion.

Flotation profiles for GLUT1 on density gradient centrifugation of mβCD-treated cells revealed a broad shift to the lower fractions without an effect on the distribution of caveolin-1 and IAP (Fig. 6D).

3.6. Immunofluorescence analysis of the membrane distribution of glucose transporters by confocal laser scanning microscopy

To determine if there is any difference in the distribution of GLUT1 and GLUT3 in living cells, immunofluorescence analyses were performed with a confocal laser scanning microscope. When CGL4/Glut3 cells, which were fixed and permeabilized at room temperature (20–24 °C), were

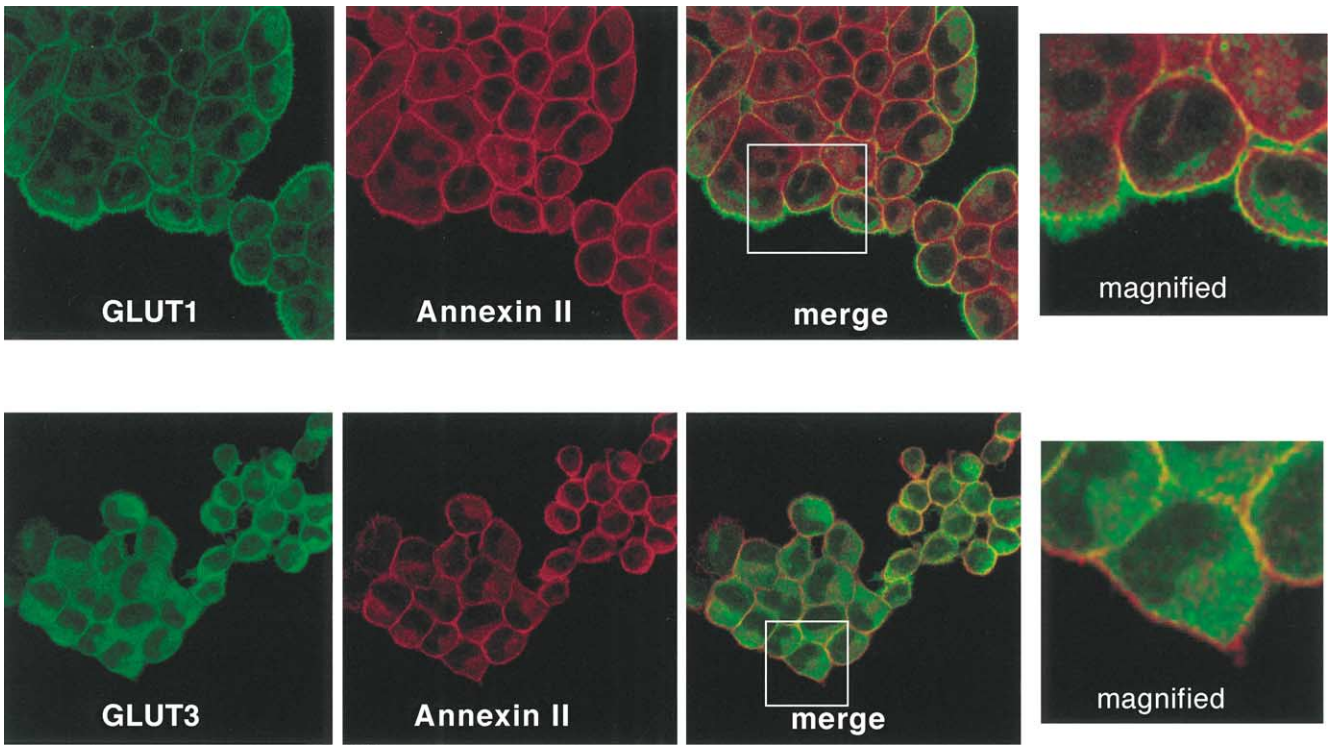

Fig. 7. Immunofluorescence detection of glucose transporters in HeLa cell lines. CGL4/Glut3 cells were fixed and permeabilized at room temperature. After staining of the cells with anti-GLUT1 or anti-GLUT3 antibody (rabbit) together with anti-annexin II antibody (mouse), followed by incubation with Alexa 488-conjugated anti-rabbit IgG or Alexa 594-conjugated anti-mouse IgG antibody, the immunofluorescence was visualized by confocal laser scanning microscopy and the two-color digital images were overlaid and processed.

stained with either anti-GLUT1 or GLUT3 antibody and an immunofluorescent-secondary antibody, both isoforms were detected along with the plasma membranes. However, the digital images for GLUT1 and GLUT3 seemed to be distinctive especially at the edge regions of the cells (Fig. 7). When each image was merged with that of anti-Annexin II, which is a calcium/phospholipid-binding protein [33] and was entirely distributed on the membrane, the preferential distribution of GLUT1 to the edge regions was found to be remarkable in these cells.

4. Discussion

It has been documented that microdomains, such as DRMs or lipid rafts, exist in the plasma membrane of various types of living cells [21–26]. In certain cells, small invaginations of the membrane, caveolae whose major component is caveolin, with a detergent-resistant phenotype are also identified. Due to the ordered nature of these microdomains, isolation with non-ionic detergents is commonly used, although more sophisticated procedures without detergents have recently been developed [26,27]. DRM- or raft-associated proteins, such as GPI-anchored proteins, Src-kinases and Ras, are organized into this lipid bilayer either through their GPI moieties or covalently linked acyl chains. The association with the DRM of caveolin, which is not anchored by GPI, does not require acylation [34].

In the present paper, we described that the distribution of glucose transporter isoforms GLUT1 and GLUT3 in the plasma membranes of non-polarized cells seems to be different. In HeLa cell lines and CHO cells, GLUT1 is distributed to detergent-resistant raft-like domains (DRM), whereas GLUT3 is preferentially distributed in fluid lipid domains. This interpretation is based upon the following results: first, GLUT3 was fully solubilized by cold non-ionic detergents, such as Triton X-100 and Lubrol WX, whereas GLUT1 remained in the insoluble fractions together with DRM-associated proteins, such as caveolin-1 and IAP (Figs. 1–3). Second, this insolubility of GLUT1 in Triton X-100 was reduced by cholesterol depletion (Fig. 6), which may control the liquid-ordered state of DRM [21–24]. Third, GLUT1 floated up to the lower density fraction containing caveolin-1, IAP and the ganglioside GM1 on density gradient centrifugation of cell lysates, whereas GLUT3 remained in the bottom layers together with most solubilized proteins including TfR and α-tubulin (Fig. 4). Finally, the differential distribution of glucose transporter isoforms in membrane domains might be due to intrinsic properties, since overproduction or N-glycosylation state did not affect the distribution, and it was seen in other cell types or tumor-suppressed hybrid cells (Figs. 2 and 3). Immunofluorescence

analysis by confocal laser scanning microscopy supports a distinctive localization of these isoforms on the living cell surface, although the linkage to the DRM structure remains unknown.

The membrane organization of DRMs or rafts would be regulated by a selective TGN-dependent trafficking of membrane components [25]. A well-defined example is the apical sorting of GPI-proteins and glycolipids to DRM domains in polarized epithelial cells. A similar TGN-dependent pathway to DRM domains may exist in non-polarized cells [25]. It seems contradictory, however, that GLUT3, appearing on the apical surface, was sensitive to detergent but GLUT1, sorted to the basolateral surface, was associated with the DRM. Although N-glycan is proposed as one of the apical sorting signals in polarized cells [25], the N-glycosylation state of the glucose transporters might not be critical to the differential distribution to the membrane domains in non-polarized cells. These observations would lead one to ask if there is some common element(s) or signal(s) for the sorting and distribution of these glucose transporters to the microdomains of membranes. More recently, a distribution to the DRM domains has been reported for P-glycoprotein [35,36], a multiple membrane-spanning protein, which mediates an ATP-dependent efflux of chemotherapeutic agents. Since no functional amino acid sequence for GPI-anchoring or the acylation of P-glycoprotein or GLUT1 has been noted, these N-glycoproteins containing 12 membrane-spanning segments may be distributed to the DRM through hydrophobic interactions with a liquid-ordered lipid phase.

Among DRMs or lipid rafts, there are distinctive microdomains, that would be regulated, at least in part, by membrane cholesterol [21–24]. On the neuronal surface, different GPI proteins, such as Thy-1 and prion protein (PrP), are distributed to different DRM domains [37]. Thy-1 localizes in a cholesterol/sphingolipid-rich ordered domain, while PrP is present in a semi-ordered lipid domain. An apical membrane protein, prominin, also exists in a distinct DRM in microvilli of epithelial cells, which is based on cholesterol content [38]. Prominin is well solubilized by cold Triton X-100, but is resistant to Lubrol WX probably due to its greater HLB than Triton X-100. In the present study, the insolubility of GLUT1 was greater in Lubrol WX than in Triton X-100 (Figs. 1 and 3). Under these conditions, caveolin-1 and IAP were insoluble, but the insolubility of these proteins in detergent was affected differently by temperature and cholesterol. GLUT1 was rendered soluble by increased temperature (Fig. 5) or the depletion of cholesterol (Fig. 6). In contrast, little increase in the solubility of caveolin-1 and IAP was caused by these treatments. These results suggest that these proteins are localized in highly ordered DRM domains, whereas GLUT1 is organized in a cholesterol-rich, but less-ordered lipid domain. In contrast, GLUT3 and TfR may exist in more fluid membrane domains.

However, a major concern about the physiological relevance of the present findings is that at the physiological temperature (37 °C) GLUT1 was extractable with Triton X-100 (Fig. 5). The increased solubility by non-ionic detergents at higher temperature is one of the well characterized features of raft-associated proteins and is ascribed to a decrease in the ordered phase of the lipid bilayer, as described for cholesterol depletion [21–24,26]. These criteria are fundamentally supported by a possible raft association of GLUT1, as described above. Evidence has also emerged that raft-associated proteins with a detergent-resistant nature are distributed to cholesterol-rich microdomains under physiological conditions [26,27]. It remains possible that the present characteristics of GLUT1 and GLUT3 are artifacts of the preparation of DRM. In line with this, we used an alternative method to isolate raft-like membranes with detergent-free medium containing a high concentration of sodium carbonate. As previously described [39], caveolin-rich membrane fractions were separated from most cellular proteins such as α-tubulin and annexin II by density gradient centrifugation. GLUT1 was retained in this caveolin-rich fraction, but it was inseparable from GLUT3 under the conditions (data not shown). The physiological relevance of the association of GLUT1 with the DRM must await further experimentation, clarifying the discrepancy over membrane components due to different procedures.

With this respect, an alternative mention of the present study may well be linked to the membrane localization of both isoforms described in polarized cells such as Caco-2 and MDCK cells, in which GLUT1 is at the basolateral surface, whereas GLUT3 is at the apical surface [17,18]. It is possible that at 4 °C, GLUT1 becomes associated with the DRM area probably due to biophysical interactions of membrane lipids and proteins. One interpretation is that these changes may be induced at the basolateral membrane, while not occurring in the apical membrane. Such a difference in the physicochemical nature of the polarized membranes or DRM domains may cause the difference in distribution and solubility at low temperature of these isoforms. These characteristics may partly explain distinctive localization of these isoforms, as revealed by immunofluorescence confocal microscopy. Future studies on the molecular mechanisms that control the localization and function of glucose transporters in DRM or non-DRM domains should clarify sorting signals and distinctive roles for these proteins in mammalian cells.

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References

[1] G.I. Bell, C.F. Burant, J. Takeda, G.M. Gould, J. Biol. Chem. 268 (1993) 19161–19164.
[2] H.G. Joost, B. Thorens, Mol. Membr. Biol. 18 (2001) 247–256.
[3] J.S. Flier, M.M. Mueckler, P. Usher, H.F. Lodish, Science 235 (1987) 1492–1495.
[4] T. Kitagawa, M. Tanaka, Y. Akamatsu, Biochim. Biophys. Acta 980 (1989) 100–108.
[5] T. Kitagawa, A. Masumi, Y. Akamatsu, J. Biol. Chem. 266 (1991) 18066–18071.
[6] T. Yamamoto, Y. Seino, H. Fukumoto, G. Koh, H. Yano, N. Inagaki, Y. Yamada, K. Inoue, T. Manabe, H. Imura, Biochem. Biophys. Res. Commun. 170 (1990) 223–230.
[7] T. Kayano, H. Fukumoto, R.L. Eddy, F.-S. Fan, M.G. Byers, T.B. Shows, G.I. Bell, J. Biol. Chem. 263 (1988) 15245–15248.
[8] S. Nagamatsu, H. Sawa, A. Wakizaka, T. Hoshino, J. Neurochem. 61 (1993) 2048–2053.
[9] M. Younes, R.W. Brown, M. Stephenson, M. Gondo, P.T. Cagle, Cancer 80 (1997) 1046–1051.
[10] M.O. Carayannopoulos, M.M.-Y. Cui, J.M. Pingsterhaus, R.A. McKnight, M. Mueckler, S.U. Devaskar, K.H. Moley, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 7313–7318.
[11] T. Kitagawa, Y. Tsuruhara, M. Hayashi, T. Endo, E.J. Stanbridge, J. Cell Sci. 108 (1995) 3735–3743.
[12] M. Noto, A. Iwazaki, J. Nagao, Y. Sumiyama, J.L. Redpath, E.J. Stanbridge, T. Kitagawa, Biochem. Biophys. Res. Commun. 240 (1997) 395–398.
[13] T. Suzuki, A. Iwazaki, H. Katagiri, Y. Oka, J.L. Redpath, J.E. Stanbridge, T. Kitagawa, Eur. J. Biochem. 262 (1999) 534–540.
[14] P.J. Saxon, E.S. Srivatsan, E.J. Stanbridge, EMBO J. 5 (1986) 3461–3466.
[15] G.M. Gould, H.M. Thomas, T.J. Jess, G.I. Bell, Biochemistry 30 (1991) 5139–5145.
[16] T. Asano, H. Katagiri, K. Takata, K. Tsukuda, J.-L. Lin, H. Ishihara, K. Inukai, H. Hirano, Y. Yazaki, Y. Oka, Biochem. J. 288 (1992) 189–193.
[17] D.S. Harris, J.W. Slot, H.J. Geuze, D.E. James, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 7556–7560.
[18] W.S. Pascoe, K. Inukai, Y. Oka, J.W. Slot, D.E. James, Am. J. Physiol. 271 (1996) C547–C554.
[19] H.F.G. Heijnen, V. Oorschot, J.J. Sixma, J.W. Slot, D.E. James, J. Cell Biol. 138 (1997) 323–330.
[20] G. Thoidis, T. Kupriyanova, J.M. Cunningham, P. Chen, S. Cadel, T. Foulon, P. Cohen, R.E. Fine, K.V. Kandror, J. Biol. Chem. 274 (1999) 14062–14066.
[21] D.A. Brown, E. London, Biochem. Biophys. Res. Commun. 240 (1997) 1–7.
[22] K. Simons, E. Ikonen, Nature 387 (1997) 569–572.
[23] R.G. Anderson, Ann. Rev. Biochem. 67 (1998) 199–225.
[24] D.A. Brown, E. London, J. Biol. Chem. 275 (2000) 17221–17224.
[25] P. Keller, K. Simons, J. Cell Sci. 110 (1997) 3001–3009.
[26] K. Simons, D. Toomre, Nat. Rev. 1 (2000) 31–39.
[27] D.A. Zacharias, J.D. Violin, A.C. Newton, R.Y. Tsien, Science 296 (2002) 913–916.
[28] K.M. Latham, E.J. Stanbridge, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 1263–1267.
[29] T. Suzuki, Y. Suzuki, K. Hanada, A. Hashimoto, J.L. Redpath, J.E. Stanbridge, M. Nishijima, T. Kitagawa, J. Biochem. 124 (1998) 383–388.
[30] N. Naslavsky, R. Stein, A. Yanai, G. Friedlander, A. Taraboulos, J. Biol. Chem. 272 (1997) 6324–6331.
[31] M. Fukasawa, M. Nishijima, H. Itabe, T. Takano, K. Hanada, J. Biol. Chem. 275 (2000) 34028–34034.
[32] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[33] P. Raynal, H.B. Pollard, Biochim. Biophys. Acta 1197 (1994) 63–93.
[34] D.J. Dietzen, W.R. Hastings, D.M. Lublin, J. Biol. Chem. 270 (1995) 6838–6842.
[35] Y. Lavie, G. Fiucci, M. Liscovitch, J. Biol. Chem. 273 (1998) 32380–32383.
[36] M. Demeule, J. Jodoin, D. Gingras, R. Béliveau, FEBS Lett. 466 (2000) 219–224.
[37] N. Madore, K.L. Smith, C.H. Graham, A. Jen, K. Brady, S. Hall, R. Morris, EMBO J. 18 (1999) 6917–6926.
[38] K. Röper, D. Corbeil, W.B. Huttner, Nat. Cell Biol. 2 (2000) 582–592.
[39] K.S. Song, S. Li, T. Okamoto, L.A. Quilliam, M. Sargiacomo, M.P. Lisanti, J. Biol. Chem. 271 (1996) 9690–9697.