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The hepatocyte glucose-6-phosphatase subcomponent T3: its relationship to GLUT2☆

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Abstract

Glucose-6-phosphatase (G6Pase) is a multiple protein complex in the endoplasmic reticulum (ER) that includes a mechanism (known as T3) for glucose exit from the ER to the cytosol. The molecular identity of T3 is not known. T3 has been shown to be functional in the absence of GLUT2, indicating that it is not GLUT2. Here we found a 55-kDa protein in high-density microsomal fraction (HDM) of rat hepatocytes that is recognized by polyclonal GLUT2 antibody raised against the GLUT2 C-terminal 14-amino-acid-sequence peptide. HDM contained calnexin but no integrin-β1 or Na/K ATPase in Western blotting. Significant GLUT2 immunoreactivity was colocalized with colligin, an ER marker, in confocal microscopy. Furthermore, the 55-kDa protein in HDM was labeled with a covalently reactive, impermeable glucose transporter substrate, 1,3-bis-(3-deoxy-D-glucopyranose-3-yloxy)-2-propyl 4-benzoyl-benzoate (B3GL) when hepatocyte homogenates, but not intact cells, were labeled. In addition glucose efflux from HDM vesicles was sensitive to B3GL treatment in a dose-dependent manner. Based on these findings, we suggest that T3 may be a novel facilitative glucose transporter that is highly homologous to GLUT2 in the C-terminal sequence, thus cross-reacting with the GLUT2 antibody. The finding will be useful in molecular identification and cloning of T3.

Keywords: GLUT2; Endoplasmic reticulum; Glucose transporter; Glucose-6-phosphatase; Hepatocyte

1. Introduction

A family of intrinsic membrane proteins (known as facilitative glucose transporters or GLUTs) catalyzes the glucose transport across the plasma membrane diffusion barrier in animal cells [1]. GLUT2, an isoform of this family, is selectively expressed in hepatocytes, pancreatic beta cells, and absorptive renal and intestinal epithelial cells [2]. The rate of glucose uptake in these cells, including hepatocytes, greatly exceeds the rate of glucose metabolism, thus GLUT2 at the cell surface does not appear to regulate the overall glucose metabolism in these cells. On the other hand, mutations in GLUT2 gene encoding nonfunctional products have been identified in association with Fanconi-Bickel syndrome [3], suggesting that GLUT2 may indeed play a role in hepatic glucose metabolism (also see below).

The liver plays a key role in the glucose homeostasis; it works as blood glucose "buffer", taking up or releasing glucose depending on the level of blood glucose [4–6]. When glucose concentration in blood reaches 6 mM, the liver takes up glucose and converts it to glucose-6-phosphate (G6P). The liver can store a large amount of glucose as glycogen but uses little glucose. During muscular activity or after an overnight fasting, the blood glucose level drops to 4 mM, the liver now produces glucose by glycogenolysis or gluconeogenesis and releases it into the blood. G6P occupies a central crossroad position in these processes. It is produced from glucose as the first step of glycolysis and glycogenesis, the reaction catalyzed by glucokinase. Conversely, G6P is converted to glucose as the terminal step of gluconeogenesis and glycogenolysis. Glucose-6-phosphatase (G6Pase) catalyzes this conversion. This conversion is an essential step in glucose homeostasis, as only glucose, but not G6P, can leave the liver into the blood stream, the process mediated by

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GLUT2. It is noteworthy in this regard that G6Pase is selectively abundant in tissues where GLUT2 is expressed [6]. The importance of hepatic glucose production in glucose homeostasis has been well documented [7–9].

G6Pase is an integral membrane protein with its catalytic activity residing in the lumen of endoplasmic reticulum (ER) [10,11]. Its physiological function requires at least five separate gene products [12], including transporters for G6P (T1), pyrophosphate (T2) and glucose (T3). Of these, T1 has been cloned [13]. This gene was found to be mutated in glycogen storage disease type 1b patients whose G6P transport function in ER was defective [9]. T3 mediates glucose exit from the lumen of the ER back to the cytosol [10]. Molecular identity of T3 is not known. GLUT2 is the isoform presently identifiable in rat hepatocytes [2,3]. A recent GLUT2 gene knock-out study [14] has shown that the absence of GLUT2 expression does not affect hepatic glucose production, suggesting that T3 is not GLUT2. This led the authors to propose a nonconventional, vesicle-mediated, glucose transport mechanism for glucose release from hepatocyte ER to the blood. The proposal, however, does not rule out the possibility that T3 may be a novel GLUT isoform operating in the ER.

In subcellular fractionation followed by Western blot, we have previously found that a GLUT2 antibody raised against the peptide corresponding to the GLUT2 C-terminal 14-amino-acid sequence recognizes a 55-kDa protein not only in a plasma membrane-containing fraction but also in a microsomal fraction of rat hepatocytes [15]. In the present study, using an improved fractionation protocol, and assessing subcellular distribution of several organelle-specific markers, we now show that this GLUT2 immunoreactivity detected in the microsomal fraction is not due to the plasma membrane GLUT2 contamination but represents a protein in the ER per se. This conclusion is also supported by immunofluorescence confocal microscopy. More importantly, we also show that a 55-kDa protein in the ER-enriched microsomal fraction is labeled with a covalently active, membrane-impermeable, glucose analog, B3GL, in cell homogenates, but not in intact cells. Furthermore, we show that the HDM vesicles transport glucose that is sensitive to B3GL treatment in a dose-dependent manner. Together, these findings strongly suggest that T3 may be a novel facilitative GLUT isoform immunologically related to GLUT2. The covalent labeling of a 55-kDa microsomal protein with B3GL together with its apparent cross-reactivity to the GLUT2 antibody would facilitate identification and cloning of T3.

2. Materials and methods

2.1. Materials

GLUT2 antibody was from Biogenesis (Brentwood, NH) (Cat. No. RAGLUT2; Batch No. 160708B). Integrin-β1 and calnexin antibody (N-terminus) were from Transduction Laboratories (Lexington, KY). Na/K ATPase antibody was from Upstate Technology (Lake Placid, NY). G6Pase antibody, a polyclonal antibody raised against the catalytic portion of G6Pase [7], was a generous gift from Dr. Rebecca Taub, University of Pennsylvania, Philadelphia, PA. Calnexin antibody (C-terminus) was from StressGene Biotechnologies (Victoria, BC, Canada). Colligin antibody was from StressGene Biotechnologies. Rabbit IgG and agarose bound protein beads were from Vector Laboratories (Burlingame, CA). Horseradish peroxidase (HRP)–protein A was purchased from Zymed (San Francisco, CA). Trisacryl bead (GF-2000) was obtained from Pierce (Rockford, IL). ECL reagent was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Octylglucoside and Triton X-100 were from Sigma (St. Louis, MI). 1,3-Bis-(3-deoxy-D-glucopyranose-3-yloxy)-2-propyl 4-benzoyl-benzoate (B3GL) was synthesized in our laboratory [16]. [^{3}H]-D-glucose was purchased from ICN Pharmaceuticals (Irvine, CA). Cellulose acetate/nitrate filters (0.22 μm pore) were from Millipore. All other reagents were from sources stated below and were of reagent grade.

2.2. Isolation of hepatocytes

Rats (Sprague–Dawley, male, 150–200 g body weight) were anesthetized by sodium pentobarbital (40 mg/kg) intramuscular injection, and the liver was perfused first with 400 ml Ca^{2+}-free Hank's buffer (pH 7.4) containing EGTA (5 mM) and D-glucose (5.5 mM) for 20 min, then with 100 ml of Hank's buffer containing $CaCl_2$ (2 mM) and collagenase (100 mg/150 ml). The liver was removed, incubated with collagenase in 50 ml of buffer at 37 °C for 10 min, and cells were filtered through a 60-μm nylon mesh. The cell suspension was kept for 20 min at 4 °C to remove erythrocytes, washed three times with cold Hank's working solution by centrifugation at $200 \times g$ (SS-34 rotor, 1000 rpm), and cells were resuspended in Krebs–Ringer phosphate buffer containing 2 mM pyruvate and 1% BSA, with pH readjusted at 7.4. After 10 min incubation at 37 °C, cells were separated by centrifugation and resuspended into the same suspension buffer at a concentration of $1–2 \times 10^6$ cells/ml. Hepatocytes were counted by a Coulter electronic particle counter. One rat liver typically gave $10–12 \times 10^6$ isolated hepatocytes or 3–4 ml of packed cells.

2.3. Subcellular fractionation

The procedures were modified from those described previously [15,17], as follows. The hepatocytes were packed by centrifugation at 2000 rpm (SS-34 rotor) for 3 min to make 50% cell suspension in homogenizing solution containing 0.25 M sucrose and 10 mM HEPES, pH 7.4. The cell suspension was aliquoted into 1 ml in Eppendorf tubes and the cells were disrupted by passing 15 times through a 22-gauge needle with 1 ml tuberculin syringe and collected each homogenate into a 40-ml centrifuge tube. Whole

homogenate was centrifuged at 200 × *g* (SS-34 rotor, 1000 rpm) for 3 min, the supernate was saved and the unbroken cells in pellets were disrupted by the same method. This procedure was repeated four times and the pooled supernate was centrifuged at 1450 × *g* (SS-34 rotor, 3500 rpm) for 10 min. The resultant pellet was used as a fraction containing plasma membrane, nucleus, and mitochondria (PM/NM). The supernate was further subjected to centrifugation at 25,000 × *g* (SS-34 rotor, 14,500 rpm) for 10 min, and the resulting pellet was used as a fraction enriched with lysosomes (LYSO). The 25,000 × *g* supernate was centrifuged at 48,000 × *g* (50.2 rotor, 23,000 rpm) for 30 min and the pellet was used as high-density microsomal fraction (HDM). The 48,000 × *g* supernate was then centrifuged at 200,000 × *g* for 180 min to obtain a pellet of low-density microsomal fraction (LDM).

2.4. Photoaffinity labeling with biotin-B3GL

Biotin-B3GL was prepared from B3GL by introducing biotin via condensation of *N*-Fmoc-biocytin *N*-hydroxysuccinimidyl ester with 2-amino-1,3-di-(1,2,4,5-diisopropylidine-3-oxyl)-propane. After deprotecting, reaction with succinimidyl ester of 4-benzoylbenzoic acid followed by removal of isopropylidine protecting groups gave the biotinylated analog (TLC by UV absorption, charring, reducing sugar and presence of biotin; Rf = 0.43).

For labeling intact cells, hepatocytes (20–25% cytocrit) were incubated for 30 min at 37 °C in Krebs Ringer Phosphate (KRP) buffer (128 mM NaCl, 5.2 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.29 mM KH_2PO_4, and 10 mM Na_2HPO_4, pH 7.4) containing 0.1% BSA and 2 mM glucose or 1 mM pyruvate. For labeling broken cells, cells were first homogenized as above in homogenizing solution containing 0.25 M sucrose and 10 mM HEPES, pH 7.4. The cell suspension or cell homogenates were then chilled in cold BSA-free buffer (4 °C) mixed with 3.5 mM biotin-labeled B3GL and 33 mM B3GL in a polypropylene dish. The mixture was immediately irradiated on ice three times for 20 s each with a 5-s intermission in between to swirl the cell suspension gently. A 450-W mercury arc lamp (Conrad-Hanovia, Newark, NJ) was used for irradiation at a distance of 6.5 cm from a silica sleeve. A filter (C57-54, Corning glass) was used to cut off radiation wavelength less than 280 nm. Following irradiation, the cells were immediately washed twice with a 20-fold volume of prechilled, B3GL-free incubation buffer to remove unbound biotin-B3GL. The procedures are modified from those described previously [16,18]. For glucose flux experiments, HDM vesicles were treated as above without (control vesicles) or with a varying concentration of B3GL with no biotin-B3GL.

2.5. Immunofluorescence confocal microscopy

The 5- to 10-mm-thick frozen sections of rat liver were treated with 2% paraformaldehyde in PBS buffer (pH 7.4) for 10 min, and subjected to a 10-min permeabilization with 0.1% Triton X-100, as described previously [8]. The slides were washed in PBS buffer and incubated sequentially with primary antibodies (rabbit polyclonal anti-Glut2 antibody and mouse monoclonal anti-colligin antibody or Glut2 and mouse monoclonal anti-integrin-β1 antibody), followed by Texas-red-conjugated goat anti-rabbit and FITC-conjugated donkey anti-mouse antibodies for GLUT2/colligin). After the sections were washed with PBS buffer containing Triton X-100 and covered with Prolong Antifade Reagent, they

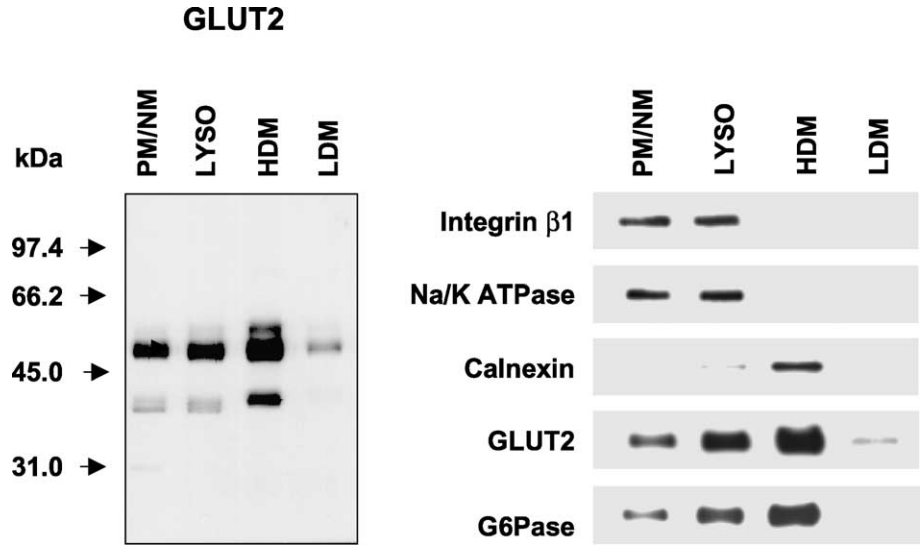

Fig. 1. GLUT2, Na/K ATPase, integrin-β1, calnexin, and G6Pase Western blots of rat hepatocyte subcellular membrane fractions. Antibodies were used after dilution in 1% milk/TTBS as specified in parentheses, and are anti-mouse integrin-β1 (2500 × dilution); anti-rat Na/K ATPase α1 (rabbit polyclonal IgG) (10,000 × dilution); anti-mouse calnexin (C-terminus) (1000 × dilution); anti-rabbit GLUT2 polyclonal antibody (750 × dilution); and anti-rabbit G6Pase (750 × dilution) times in 1% milk/TTBS. Each lane contained 20 mg of membrane protein. The results were reproduced in three independent experiments.

were visualized using a scanning laser confocal immunofluorescence microscope.

2.6. Gel electrophoresis and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described [19] with a slight modification as follows. Proteins were solubilized with sample buffer containing 0.5% mercaptoethanol for 30 min at room temperature and applied 10–20 μg of protein onto 10% acrylamide gel. Gels were run with 150 constant volts, and transferred to NC membrane for 1.5–2 h with 105 constant volts. The NC membranes were then blocked with 5% milk in TBS (50 mM Tris-base, 150 mM NaCl) for 30 min at room temperature with shaking, and incubated overnight with primary antibody in 1% milk/TTBS (TBS + 0.1% Tween 20). The membranes were washed three times with TTBS for 10 min each, and incubated with secondary antibody (HRP–protein A) in 1% milk/TTBS for 30 min at room temperature with shaking. The membrane was then reacted with ECL reagent for 1 min and exposed to X-ray film at dark room. The X-ray films were developed at an Autodeveloper and images were quantitated by densitometer. For biotin-B3GL labeled membranes, the transferred NC membranes were incubated overnight in 5% milk/TBS, and incubated directly with streptoavidin–HRP at concentration of 0.2 μg/ml of 1% milk/TTBS for 30 min at room temperature with shaking. The membranes were washed three times with TBS for 10 min each, reacted with ECL reagent for 1 min, and processed similarly as above.

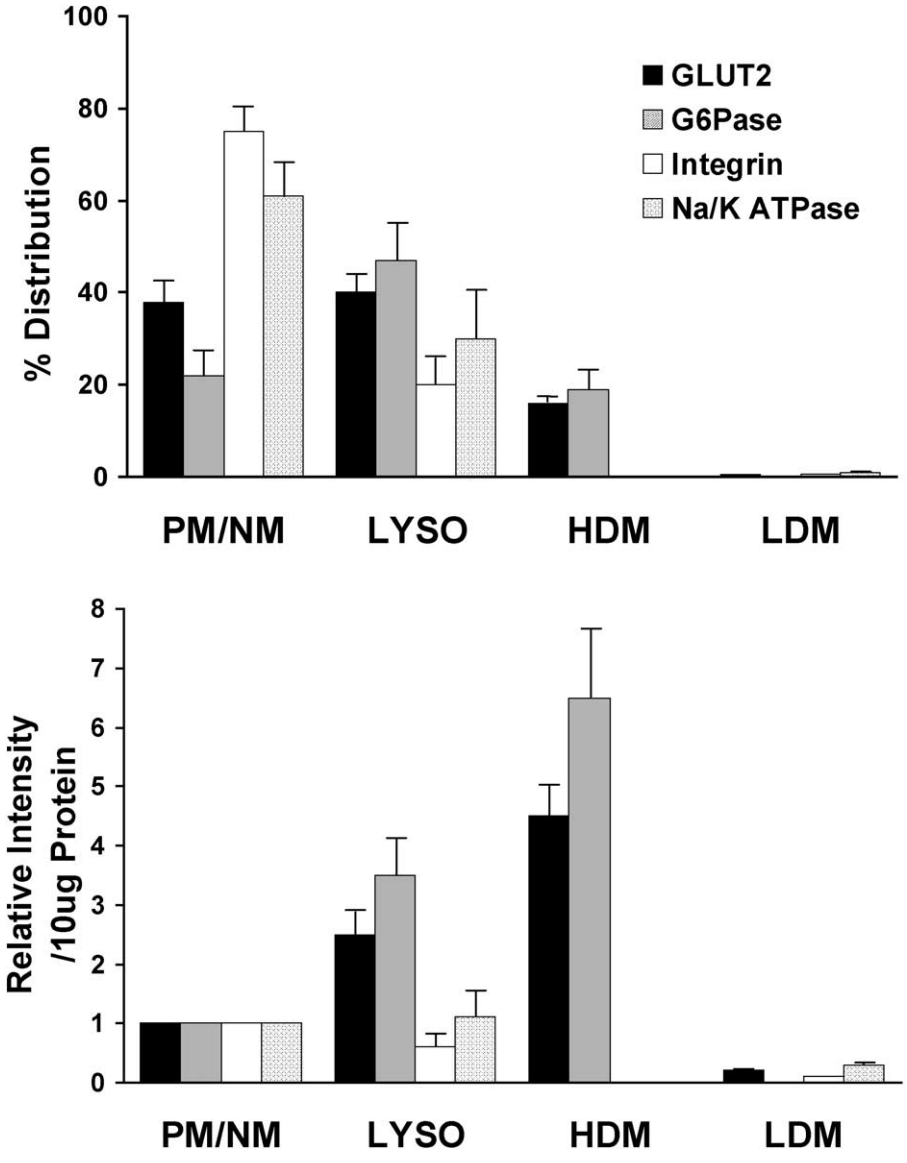

Fig. 2. Subcellular distribution of the 55-kDa GLUT2, G6Pase, integrin and Na/K ATPase. The percentage distribution of the immunoreactivities (*upper panel*) was calculated from the relative blotting intensities per unit protein mass and protein contents for each fraction. The relative blot intensities (*lower panel*) were expressed in an arbitrary unit normalized against that of PM/NM. Calnexin data were not included in this analysis as it was confined to HDM fraction.

2.7. Measurement of D-glucose flux across HDM vesicles

HDM vesicles before and after covalent labeling with B3GL were used. Glucose flux across vesicles were measured according to Banhegyi et al. [20] using Millipore filters with a minor modification. Briefly, HDM vesicles (1 mg/ml) in 10 mM HEPES buffer, pH 7.4, containing 0.25 M sucrose were preloaded with 20 mM glucose in the presence of a tracer amount of [^{3}H]glucose (10 μCi/ml) for 1 h at room temperature, recovered as pellets by centrifugation at 48,000 × *g* for 30 min, and were transferred into a 10-fold volume of glucose-free 10 mM HEPES buffer, pH 7.4, containing 0.25 M sucrose (efflux assay buffer) at time equal zero. Sample aliquots were taken at specified time intervals, filtered through Millipore filters, and quickly washed four times, each time with 2-fold volume of ice-cold, efflux assay buffer containing 2 mM mercuric chloride. The radioactivity on the filters was measured in LKB liquid scintillation counter.

3. Results

3.1. GLUT2 immunoreactivity in a plasma membrane-free microsomal fraction of rat hepatocytes

GLUT2 is responsible for the cellular uptake and release of glucose in hepatocytes [2]. Using polyclonal GLUT2 antibody raised against the GLUT2 C-terminal 14-amino-acid-sequence peptide, we have previously detected a broad 55-kDa protein band as the major immunoreactive protein in total hepatocyte membranes, with a 38-kDa protein as a minor immunoreactive protein [19]. Digestion with Endo-F/peptide-*N*-glucosidase converted the 55-kDa protein to a 38-kDa protein (unpublished observation), indicating that the 38-kDa protein is, at least in part, a nonglycosylated, immature form of GLUT2, whereas the 55-kDa protein being the fully glycosylated, matured GLUT2. Upon subcellular fractionation, both the 55-kDa and 38-kDa GLUT2 immunoreactive protein species were detected in PM/NM, LYSO and HDM fractions but not in LDM fraction (Fig. 1). Alternatively, a 38-kDa band may represent a proteolytic fragment of the GLUT2 immunoreactive 55-kDa protein.

Relative contents of selected membrane proteins in each of these subcellular membrane fractions were also assessed (Fig. 1). The two well-known plasma membrane markers, integrin-β1 and Na/K ATPase, were present in PM/NM and LYSO, but not in HDM and LDM. Calnexin, an ER marker, on the other hand, was present abundantly in HDM, only slightly if any in LYSO, and totally absent in PM/NM and LDM. Together, these findings clearly indicate that the plasma membrane is present only in PM/NM and LYSO fractions, but not in HDM or LDM. The findings also indicate that the ER is quantitatively recovered in HDM fraction, and is effectively excluded from PM/NM, LYSO, and LDM.

The detection of GLUT2 immunoreactivity in PM/NM and LYSO is expected from the presence of the two plasma membrane markers integrin-β1 and Na/K ATPase in these fractions. Our detection of abundant GLUT2 immunoreactivity in HDM, the fraction virtually free of the two plasma membrane markers (Fig. 1), however, strongly suggests that the GLUT2 immunoreactivity in HDM is not due to the plasma membrane GLUT2 contamination. Interestingly, a significant amount of G6Pase was detected in PM/NM fraction in immunoblots (Fig. 1), most likely due to apparent association of this enzyme with the nuclear envelop [21].

Semi-quantitative analyses of the blotting intensities and the percentage distribution of the 55-kDa GLUT2 and other relevant membrane proteins among different fractions are summarized in Fig. 2. Significantly, as much as 18.2 ± 1.8% (a mean ± S.D., calculated from results of four experiments) of the cellular 55-kDa GLUT2 immunoreactivity is in HDM, the fraction that is free of plasma membrane contamination. Moreover, the relative intensity of the 55-kDa GLUT2 immunoreactivity, expressed per unit protein mass (lower panel) is significantly greater in HDM than in PM/NM and LYSO, clearly indicating that the immunoreactivity in HDM is not due to possible plasma membrane contamination. Similarly, only 20% or less of the cellular G6Pase is detected in HDM, suggesting that a large portion of cellular G6Pase reside in membranes other than the ER, including

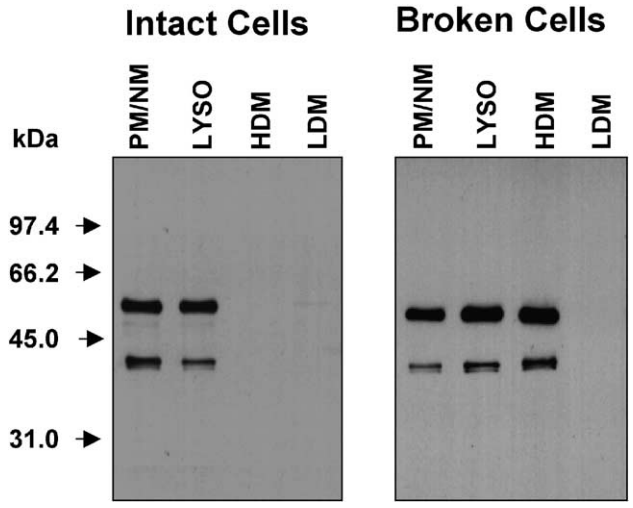

Fig. 3. Covalent labeling of hepatocyte membrane proteins with B3GL. The photolabeling procedures were essentially those used for labeling GLUT4 in rat adipocytes [22]. Hepatocytes before (intact cells) and after homogenization (broken cells) were used for the plasma membrane-selective, and total cellular GLUT2 covalent labeling, respectively. Intact cells or homogenates were suspended in an ice-cold solution of biotinylated B3GL and B3GL (in 1–10 M ratio) in TBS, and irradiated using a 450-W mercury lamp at 6.5 cm from the silica sleeve through a layer of water as heat filter. The irradiated cells were washed extensively with ice-cold TBS before homogenization and subcellular fractionation. When broken cells were labeled (broken cells), cells were homogenized without or with fractionation before photolabeling. Proteins in each subcellular fraction were resolved in SDS-PAGE and Biotin-B3GL label was quantitated based on biotin–streptavidin–HRP interactions.

the nuclear membranes [21]. The relatively low blotting intensities (per unit protein mass) of integrin-β1 and Na/K ATPase, the two plasma membrane markers tested, in PM/NM compared to LYSO and HDM, is in part due to large nuclei and mitochondria (NM) protein (65–70%) in this fraction.

3.2. A 55-kDa microsomal protein is labeled with B3GL in cell homogenates but not in intact cells

B3GL, a membrane-impermeable, photolytically active, glucose analog, has been used to label the plasma membrane GLUT4 selectively without labeling microsomal GLUT4 pool in intact rat adipocytes [16,18]. B3GL is surface-selective and not GLUT protein-selective in certain cell types; since it labels a 70-kDa membrane protein of unknown identity, in addition to GLUT protein in adipocytes [16,18]. On the other hand, B3GL labeling in human erythrocytes is specific to GLUT1; it labels a single 45-kDa membrane protein that is photolytically labeled by cytochalasin B (unpublished observation).

Rat hepatocytes were labeled with biotin-B3GL and subjected to subcellular fractionation as described in Materials and Methods. When intact cells were used for the photolysis, it resulted in labeling of 55-kDa and 38-kDa membrane proteins in PM/NM and LYSO fractions, but not in HDM fraction (Fig. 3). However, when the same labeling procedure was repeated after cells were first homogenized (broken cells), both 55-kDa and 38-kDa proteins were labeled not only in PM/NM and LYSO fractions, but also in HDM fraction as well (Fig. 3). A straightforward interpretation of these findings would be that the B3GL-labeled 55-kDa and 38-kDa proteins detected in HDM were not accessible to the B3GL labeling in intact cells, but became accessible in broken cells. This is a strong support that the labeled 55-kDa protein in HDM is not a contaminating plasma membrane protein, namely, the plasma membrane GLUT2. The electrophoretic mobility of the 55-kDa B3GL-labeled protein was indistinguishable from that of the GLUT2 immunoreactivity in each of these fractions (not illustrated). Furthermore, the relative intensities of the B3GL labeling among different fractions from broken cells

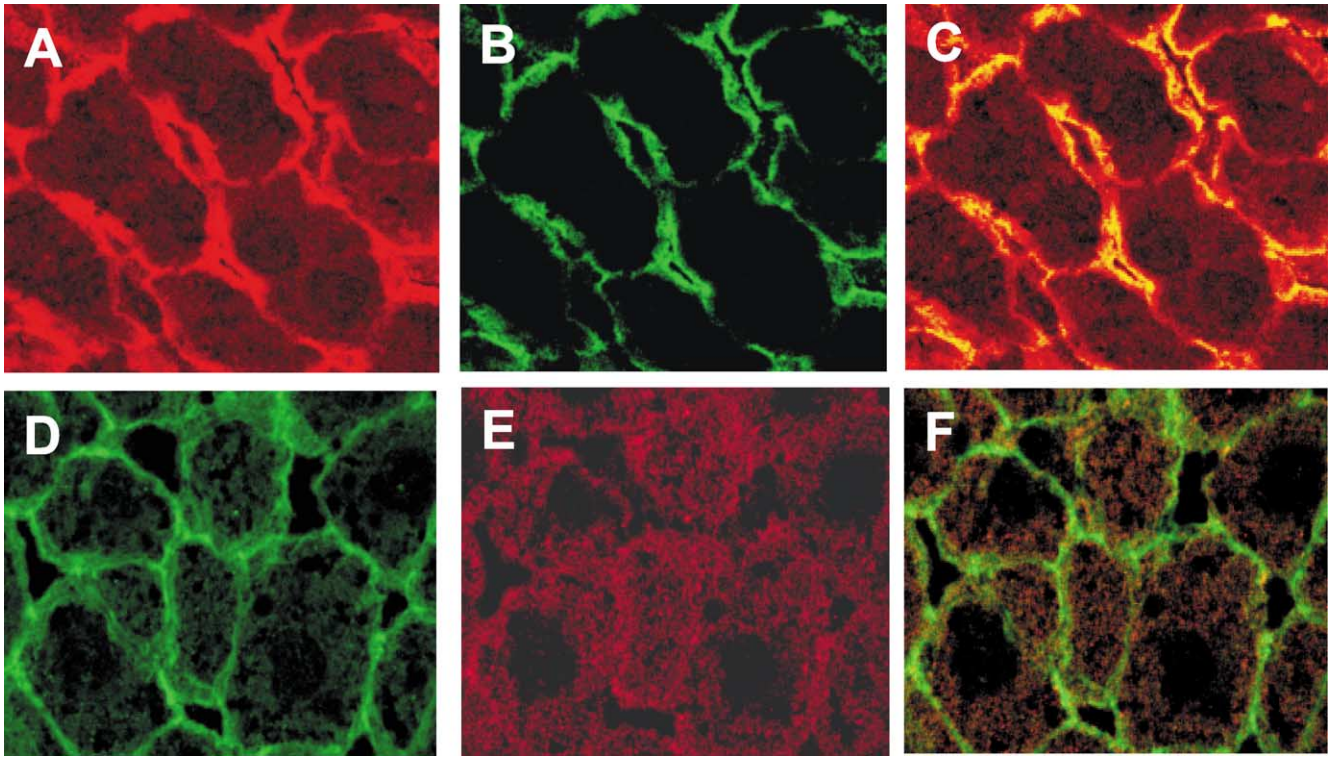

Fig. 4. Confocal images showing rat hepatocytes double immunofluorescence labeled with GLUT2 and integrin-β1 (A–C) or colligin (D–F). Cells fixed, permeabilized, and incubated with polyclonal antibody against GLUT2 (A and D) and monoclonal antibody against integrin-β1 (B) or mouse monoclonal anti-colligin antibody (E). The secondary antibodies used were Texas-red-conjugated goat anti-rabbit (A), FITC-conjugated donkey anti-mouse (B), FITC-conjugated goat anti-rabbit (D), and Cy5-conjugated donkey anti-mouse (E) antibodies. Note that GLUT2 antibody staining is evident not only at the cell surface but also with particulate structure throughout the cytoplasmic space (A and D). This is in contrast to the selective staining of the cell surface and intracellular structure by integrin-β1 (B) and colligin (E), respectively. The extensive colocalization of GLUT2 with integrin-β1 at the cell surface, but not in the cytoplasmic space, is evident and visible as yellow-orange fluorescence overlaying A and B (C). Also evident is the intracellular, but not the plasma membrane, colocalization of GLUT2 with colligin, visible as orange fluorescence by overlaying D and E (F). Each picture was delivered at 600× magnification.

are remarkably similar to the relative GLUT2 immunoblot intensities (Fig. 3), suggesting that the B3GL-labeled protein in HDM is most likely the protein recognized by the GLUT2 antibody in immunoblots.

3.3. Colocalization of GLUT2 immunoreactivity with an ER marker, colligin, in intact hepatocytes

The presence of the GLUT2 immunoreactive entity in the ER in hepatocytes is further supported by immunofluorescence confocal microscopy (Fig. 4). Integrin-β1 immunostaining was highly intense at the cell surface, but conspicuously absent throughout the intracellular space (Fig. 4, panel B). Colligin immunostaining, on the other hand, clearly revealed numerous punctuate labeling of intracellular structures with little cell surface labeling (Fig. 4, panel E). These findings confirm that integrin-β1 and colligin are selective markers for the plasma membrane and the ER, respectively. Colligin is known to reside selectively in the ER [22]. The calnexin antibody used for immunoblots did not give a good-quality immunostaining for the microscopy.

Significantly, the GLUT2 immunostaining was not only highly concentrated at the cell surface, but also to a significant extent distributed throughout the intracellular space (Fig. 4, panels A and D). This is in contrast to the total lack of integrin-β1 staining throughout intracellular space discussed above (Fig. 4, panel B), and indicates the presence of GLUT2 immunoreactivity in the intracellular compartments. The GLUT2 immunoreactivity in the ER is further confirmed by double staining for GLUT2 and colligin. Most of the intracellular GLUT2 staining was overlapped with colligin staining showing orange, while that of cell surface was not (Fig. 4, panel F). This is in contrast to the overlay of GLUT2 and integrin-β1 double labeling image (Fig. 4, panel C) where GLUT2 staining was overlapped with integrin-β1 staining at the cell surface, but not in the intracellular compartments.

3.4. Demonstration of B3GL-sensitive glucose efflux in HDM vesicles

To study transport property of T3 in HDM fraction and its relationship to the 55-kDa protein that is labeled with B3GL in cell homogenates but not in intact cells, we measured glucose efflux from glucose-loaded HDM vesicles before and after covalent labeling with B3GL (Fig. 5). HDM vesicles showed a glucose efflux that was effectively arrested by cold temperature and 2 mM mercury chloride. Although our efflux time course measurements were not rigorous enough to detect the fast and slow flux components reported previously (20), the following points are nevertheless clear and noteworthy: The flux was relatively rapid, showing a 50% net loss of glucose content in vesicles occurred at around 1 min. The efflux was also sensitive to *N*-ethylmaleimide, being inhibited by more than 80% in the presence of 5 mM *N*-ethylmaleimide. Most notably, the covalent labeling of HDM vesicles with 10 and 30 mM B3GL reduced the rate of glucose efflux significantly and in a dose-dependent manner, showing a 50% net loss at approximately 2 and 4 min, respectively. The findings are consistent with the interpretation that the glucose transport across the hepatocyte HDM vesicles is mediated by a protein, namely T3, the 55-kDa protein that was labeled by B3GL only in isolated vesicles but not in intact cells (Fig. 3).

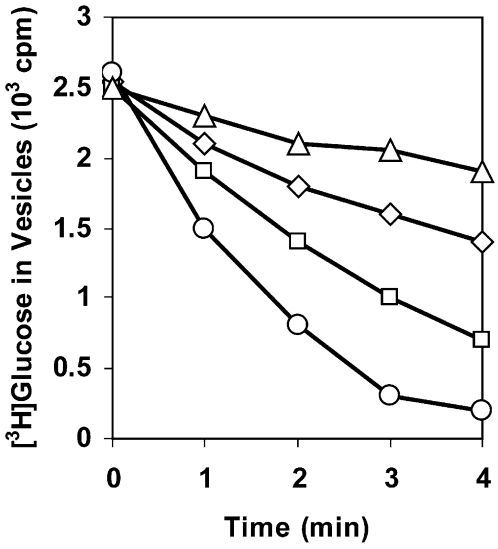

Fig. 5. Glucose efflux from glucose-preloaded hepatocyte HDM vesicles. Vesicles before and after covalent labeling with B3GL were loaded with 20 mM glucose in the presence of a tracer amount of [^{3}H]glucose. Glucose-loaded vesicles were resuspended in a glucose-free buffer at time equal zero and a 4-min time course of changes in vesicular glucose content was followed as detailed in Materials and Methods. Glucose contents in vesicles were measured based on radioactive tracer expressed in cpm on the *Y*-axis. Data are means of two to three independent measurements. Symbols are with vesicles before B3GL treatment and no additive (circles), with vesicles before B3GL treatment and in the presence of 5 mM *N*-ethylmaleimide (triangles), with vesicles after treatment with 10 mM B3GL (squares) or with 30 mM B3GL (diamonds) with no additive.

4. Discussion

Here we have shown that there is a 55-kDa protein in an ER-enriched microsomal fraction of hepatocytes that is recognized by a GLUT2 antibody and also reacts with a covalently active, glucose analog, B3GL. We also detected a 38-kDa protein as a minor species recognized by the GLUT2 antibody as well as by B3GL labeling. Although the identity of this band is unknown, the possibilities include proteolytic fragment of GLUT2 and/or unglycosylated GLUT2. The 55-kDa protein in HDM is not a contaminating plasma membrane protein, but actually resides in the ER. The following findings support this conclusion: First, the ER-enriched microsomal fraction is totally devoid of two plasma membrane markers, namely, integrin-β1 and

Na/K ATPase, but contains practically all of the cellular calnexin (Figs. 1 and 2). Second, B3GL labeling of the 55-kDa protein in the ER-enriched fraction occurs only when broken cells, but not intact cells, were used for the labeling (Fig. 3). Finally, immunofluorescence confocal microscopic findings (Fig. 4), the assay method that does not involve subcellular fractionation, thus free from homogenization artifacts, also support the presence of a significant portion of GLUT2 immunoreactivity in microsomes, most likely in the ER, in addition to the plasma membrane. Most of the cellular GLUT2 immunoreactivity is in the plasma membrane. The non-plasma membrane microsomal GLUT2 immunoreactivity amounts to 17–19% of the total cellular GLUT2 immunoreactivity (Fig. 2). The putative GLUT2 immunoreactivity in the ER could be significantly less than this estimate as immunofluorescence confocal microscopy data (Fig. 4) would suggest.

Here we have also demonstrated the presence of a protein-mediated rapid glucose flux across HDM vesicles as indicated by its sensitivity to low temperature and the inhibition by mercury chloride and *N*-ethylmaleimide (Fig. 5) and that this glucose flux is inactivated after covalent reaction with B3GL in a dose-dependent manner (Fig. 5). These findings strongly suggest that the 55-kDa protein in HDM labeled by B3GL and recognized by a GLUT2 antibody is indeed T3. As to the relationship between GLUT2 and T3, it is possible that GLUT2 is present not only at the plasma membrane but also may be present in the ER and function as T3. This possibility, however, is not supported by the recent demonstration [14] that the hepatic glucose production, including the putative T3 function [23], is normal in the absence of GLUT2 expression. It is now more likely that T3 may be a novel isoform of the facilitative GLUT family. Our demonstration that a 55-kDa protein of microsomal origin is labeled by B3GL, a covalently active GLUT substrate (Fig. 3), is clearly in favor of this possibility. The role of such a protein, on the other hand, is less obvious in the alternative glucose transport pathway involving vesicle translocation that these authors have suggested [14,23]. Recently, a number of new GLUT isoforms have been cloned [24–26], thus it is not surprising to expect a new isoform in this family. The novel GLUT isoform proposed here could be closely related to GLUT2 at least in partial amino acid sequence in its C-terminal cytoplasmic domain, as suggested by its apparent recognition by the GLUT2 antibody raised against the peptide corresponding to the GLUT2 C-terminal amino acid sequences.

Transport property of T3 has been partially characterized by studying glucose flux using hepatocyte ER-containing microsomal preparations and shown to be significantly different from that of GLUT isoforms [10,27–29]. In contrast to all other GLUT isoforms now known, T3 was shown to be not inhibited by cytochalasin B, and accepts L-glucose as a substrate. T3 was also known to show the V_{max} and K_m to glucose much larger than GLUT2. Because of the large surface area-to-volume ratio inherent to these microsomal preparations, the accurate assay for rapid flux such as that of glucose is technically difficult, and it is not known how much of these differences are real and not an experimental artifact. The rapid L-glucose flux observed also could be due to leaky microsomes. A novel approach is needed to confirm free of experimental artifact the apparent difference between T3 and GLUT properties. Although the data shown in the present study do not offer unequivocal evidence that T3 function coincides with the 55-kDa protein in HDM constituting a GLUT2 variant, they do provide critical information useful in future studies of the molecular identification and cloning of T3.

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