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Introduction and Expression of Glucose Transporters in Pancreatic Acinar Cells by *In Vivo* Electroporation

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cDNAs encoding facilitated-diffusion glucose transporters GULT1, GULT3, GULT4, and GULT5 were introduced into the rat pancreas by *in-vivo* electroporation method, and their expression and localization in pancreatic acinar cells were examined immunohistochemically. GLUT1 was localized at the basolateral membrane, whereas GLUT3 and GLUT5 were at the apical membrane. Restriction of GLUT3 and GLUT5 to the apical membrane makes a marked contrast to their localization to the entire plasma membrane when expressed in hepatocytes *in situ*. Such differential localization may be due to differential apical targeting mechanism: direct targeting in acinar cells and indirect transcytotic delivery in hepatocytes *in situ*. GLUT4 was present in the membrane of zymogen secretory granules in the cytoplasm in acinar cells. This observation suggests that GLUT4 is segregated to the regulated secretory pathway. Expression of glucose transporters *in situ* is a useful method in analyzing the targeting mechanism of membrane proteins in cells of tissues *in situ*.

Key words: glucose transporters, *in-vivo* electroporation, pancreas

I. Introduction

Glucose transporters (sugar transporters) of GLUT family are membrane proteins that facilitate the transfer of sugars across the plasma membrane. Several isoforms have been identified [2, 3, 10] and they exhibit a unique tissue-specific expression pattern [29]. GLUT1 is abundant in the blood-tissue barriers and serves in the transfer of glucose across the barrier cell layers [24, 26, 30]. GLUT2 is a low-affinity transporter and is expressed in the liver and the pancreatic B cells [33]. GLUT2 in B cells is considered to constitute a part of sensing machinery for blood glucose level and to play a role in the maintenance of blood glucose levels. GLUT3 is a neuronal glucose transporter [12]. GLUT4 is an insulin-regulatable glucose transporter, and is mainly expressed in the fat and muscle tissues [4]. GLUT5 is a fructose transporter present in the small intestine and kidney [5, 21].

In addition to differential expression, glucose transporters are localized to distinct plasma membrane domains of epithelial cells depending on the cell-types expressed [24, 34]. GLUT1 and GLUT2 are localized at the basolateral plasma membrane, whereas GULT5 is localized in the apical membrane in the kidney epithelial cells. Moreover, GULT4 resides in the intracellular compartments [4, 19, 28]. Upon insulin stimulation, it is recruited to the plasma membrane and serves in the uptake of glucose. Distinct localization of glucose transporters is crucial in the vectorial transfer of sugars in the small intestine and the kidney, and regulated glucose uptake in fat and muscle cells. These differential localization patterns of glucose transporter isoforms provide a unique opportunity to investigate whether or not a common targeting mechanism of membrane protein is working in cells comprising different parts of the body [7, 9, 23].

To shed light on the localization mechanism of glucose transporters, their cDNAs were introduced into cultured epithelial cells such as MDCK cells [13, 32], and Caco-2 cells [9, 11]. Results obtained in these cell culture systems, however, do not necessarily represent the mechanism working in the tissue cells constituting the body *in situ*. Therefore, we tried to introduce and express glucose transporters in cells of organs in the living animals. We showed previously that injection of plasmid DNA followed by electroporation in the organ of the body successfully intro-

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duced foreign genes in the rat liver [17, 22]. Using this technique we introduced glucose transporter cDNAs into the rat pancreas and examined their localization patterns immunohistochemically and compared them with those in the liver and cultured cells.

II. Materials and Methods

Animals

Male Wistar rats of 6 weeks of age supplied from the Animal Breeding Facility, Gunma University School of Medicine (Gunma, Japan) were used. All the procedures were in accord with the "Guide for the Care and Use of Laboratory Animals" of the National Academy of Sciences U.S.A., and approved by the Animal Care and Experimentation Committee, Gunma University, Showa Campus.

Plasmid DNAs of sugar transporters

The cDNA clones of rabbit GLUT1, rat GLUT3, rat GLUT4, and rat GLUT5 were as previously described [17]. The EcoRI DNA fragments (for GLUT3, GLUT4, GLUT5) and the HindIII DNA fragment (for GLUT1), all including the entire open reading frame of cDNA clones, were ligated into the pcDNA3 expression vector (Invitrogen, Groningen, The Netherlands). pEGFP-C1 was from Clontech (Palo Alto, CA). Plasmid DNAs were multiplied by culturing the host bacteria JM109 and isolated with a Qiagen plasmid DNA isolation kit (Qiagen, Santa Clarita, CA). They were dissolved in phosphate-buffered saline (PBS) at a concentration of 1 μg/μl.

In-vivo *electroporation*

Rats were divided into five groups, each consisted of three animals, and used for the transfection of plasmid DNAs harboring glucose transporters, i.e., GLUT1, GLUT3, GLUT4, and GLUT5. pEGFP-C1 was cotransfected to visualize successfully transfected cells. One group received pEGFP-C1 only and served as a control. Electric gene transfer was carried out basically as described previously [17, 22]. An Electro Square Porator (T820; BTX, San Diego, CA) with a pair of tweezer-type electrodes whose tips consisted of 1-cm-diameter disks (model 449-10 PRG; Meiwa Shoji, Tokyo, Japan) was used [22]. The electric resistance of the tissue was monitored with a graphic pulse analyzer (MVC540R, Meiwa Shoji). The rats were anesthetized with diethyl ether, and the caudal region of the pancreas received the injection of 100 μl of plasmid DNAs comprising 50 μg for glucose transporter and 50 μg for EGFP with a 27-gauge needle. Immediately afterward, the injected portion was sandwiched with a pair of tweezer-type electrode disks. Four electric pulses with 1-second interval between pulses were applied under conditions as follows: 10–50 V in voltage intensity, 10–50 ms in duration. Electroporated pancreas was moved back to the abdominal cavity and the abdominal wound was sutured.

Tissue specimens

One day after *in vivo* transfection, rats were killed under ether anesthesia. The electroporated portions of the pancreas were resected. The samples were examined with an immunofluorescence microscope for the successful transfection of pEGFP-C1. They were fixed in 3% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer, pH 7.4, for 2 hr on ice and washed with PBS. For electron microscopic immunohistochemistry, specimens were fixed on ice in 3% PFA-0.1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 for 3 hr. Specimens were infused with 20% sucrose in PBS containing 0.02% sodium azide at 4°C overnight, embedded in Tissue Tek OCT compound (Sakura Finetechnical, Tokyo, Japan), rapidly frozen in liquid nitrogen, and stored at −80°C until use. Cryostat sections of 10 μm thick were cut and mounted on poly-L-lysine-coated glass slides, and washed with PBS.

Immunofluorescence staining

Staining was carried out basically as previously described [15, 17]. In short, sections were treated with 5% normal goat serum in PBS at room temperature for 20 min. Sections were incubated with one of anti-glucose transporter antibodies at room temperature for 1 hr. Anti-glucose transporter antibodies used were rabbit anti-GLUT1 (1:500 dilution) [26], rabbit anti-GLUT3 (1:1000 dilution) [16], rabbit anti-GLUT4 (1:500 dilution) [28], and rabbit anti-GLUT5 (1:500 dilution) [9]. After being washed with PBS, the sections were incubated with LRSC (lissamine rhodamine sulfonyl chloride)-labeled donkey anti-rabbit IgG (Jackson Immunoreseach, West Grove, PA, 1:200 dilution) as a secondary antibody at room temperature for 1 hr. Two μg/mlDAPI(4',6-diamidino-2-phenylindoledihydrochloride; Boehringer-Mannheim, Germany) was included in the secondary antibody solution for DNA counterstaining [25]. After being washed with PBS, the sections were mounted in a mounting medium comprising 22% polyvinylalcohol (Wako, Japan), 11% glycerol, 56 mM Tris-HCl buffer, pH 9.0 containing 5% DABCO (1,4-diazabicyclo [2,2,2] octane) as an anti-bleaching reagent [15]. The sections were examined with an AX-70 epifluorescence microscope (Olympus, Tokyo, Japan) and images were recorded with a PXL1400 cooled-CCD camera (Photometrics, Tucson, AZ, USA). Specimens were also examined with a BX-50 microscope (Olympus) equipped with an MRC-1024 laser confocal system (Bio-Rad) utilizing a 15-mW krypton/argon laser.

Electron-microscopic immunohistochemistry

Immunostaining was carried out basically as previously described [31]. In short, cryostat sections were washed with PBS, covered with 5% normal goat serum for 10 min, and sequentially incubated with rabbit anti-GLUT4 (1:200 dilution) at room temperature overnight, and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:100 dilution, DAKO Japan, Kyoto, Japan) at room temperature for 2 hr. They were washed with PBS, immersed in a mixture of

0.06% 3.3'-diaminobenzidine (DAB) and 0.03% H_2O_2, washed with water, and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Specimens were osmicated, dehydrated with a graded series of ethanols, and embedded in epoxy resin. Ultrathin sections were cut and examined with a JEM-1010 electron microscope (JEOL, Tokyo, Japan).

III. Results

Introduction of EGFP by in-vivo *electroporation*

To optimize the condition of *in vivo* electroportation in the rat pancreas, 100 μg of pEGFP-C1 was injected into the pancreas, and received electroporation procedures of various voltage and duration. We tested the combination of voltage and duration as follows: 50 V/50 msec, 25 V/25 msec, 20 V/20 msec, and 10 V/10 msec. The specimens were processed as described in Materials and Methods, and the fluorescence of EGFP and the tissue damages by electroporation were assessed with fluorescence and Nomarski images. At 50 V/50 msec, severe damage in the acinar tissues was observed. At 10 V/10 msec, little tissue damage was seen, but expression of EGFP was scarce. Injection of the plasmid without electroporation resulted in no expression of EGFP. Considering the tissue damages and the efficiency of EGFP expression, we used a condition of 20 V/20 msec for the introduction of glucose transporters in the present study.

Localization of glucose transporters transfected in vivo

Rat pancreases were transfected with plasmids harboring glucose transporters, GLUT1, GLUT3, GLUT4, and GLUT5 by the *in-vivo* electroporation method. To facilitate identifying the transfected cells, EGFP was cotransfected. Twenty-four hours after the transfection, the expression of EGFP was clearly observed and used to identify the transfected cells. After immunofluorescence staining for glucose transporters, fluorescence microscopic images for EGFP and glucose transporters as well as the Nomarski images of acini were recorded. The isoform-specific localization of glucose transporters to distinct membranes was highly reproducible as long as the integrity of acini was maintained.

Exocrine pancreas is composed of many acini where acinar cells synthesize, store, and secrete hydrolytic enzymes of the pancreatic juice. Pancreatic acinar cells are highly polarized epithelial cells whose plasma membrane is composed of the tiny apical membrane facing the narrow central lumen and the broad basolateral membrane. Tight junctions separate these two distinct membrane domains. GLUT1 was localized to the basolateral membrane in the aciar cells (Fig. 1a, b). GLUT3 was confined to the apex of the acinar cells (Fig. 1c, d). Lateral and basal aspects were not labeled. Positive signal for GLUT4 was found intracellularly (Fig. 1e, f). It was preferentially localized to the supranuclear part of the cytoplasm, just underneath the apical membrane where most of the zymogen granules resided. Basal part of the cytoplasm was scarcely labeled for GLUT4. GLUT5 was present in the apical membrane facing the lumen (Fig. 1g, h). Basolateral membrane was not labeled. The localization pattern of glucose transporters in the pancreatic aciar cells is summarized in Table 1.

To further analyze the localization of GLUT4, electronmicroscopic immunohistochemistry was performed by the HRP-labeling preembedding method. Expressed GLUT4

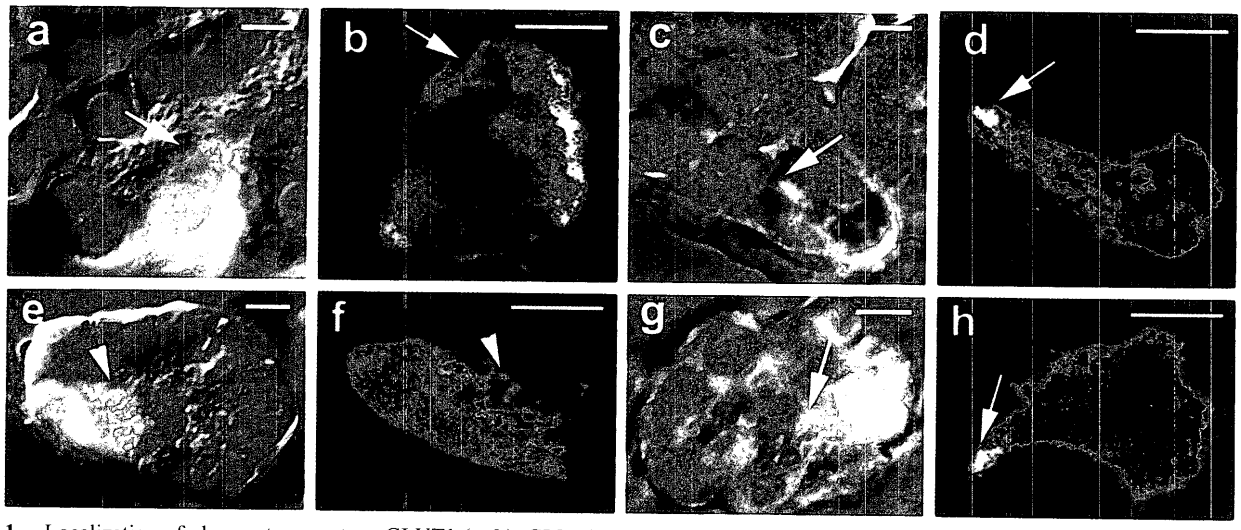

Fig. 1. Localization of glucose transporters, GLUT1 (**a, b**), GLUT3 (**c, d**), GLUT4 (**e, f**), and GLUT5 (**g, h**), introduced into pancreatic acinar cells *in situ*. Glucose transporters, EGFP, and DNA are shown in red, green, and blue, respectively. Bars=5 μm. Conventional fluorescence images projected onto Nomarski images (**a, c, e, g**) and enlarged confocal images of corresponding positive cells (**b, d, f, h**) are shown. **a, b:** GLUT1 is present along the basolateral membrane of acinar cells. Apical membrane (arrows) is not labeled. **c, d:** GLUT3 is present in the apical aspect (arrows) of the acinar cell. **e, f:** GLUT4 is retained in the supranuclear region (arrowheads) of the acinar cell. **g, h:** GLUT5 is present in the apical aspect (arrows) of the acinar cell.

Table 1. *Comparison of the localization of glucose transporters expressed in cultured epithelial cells, acinar cells in the pancreas, and hepatocytes in the liver*

glucose transporter isoform	MDCK and Caco-2 cells*	localization acinar cells in the pancreas	hepatocytes in the liver**
GLUT1	basolateral	basolateral	basolateral
GLUT3	apical	apical	apical>basolateral***
GLUT4	intracellular	intracellular	intracellular
GLUT5	apical	apical	apical>basolateral***

* [7, 9, 11, 13]
** [17]
*** Localization to both the apical and basolateral membrane with significant enrichment at the apical membrane.

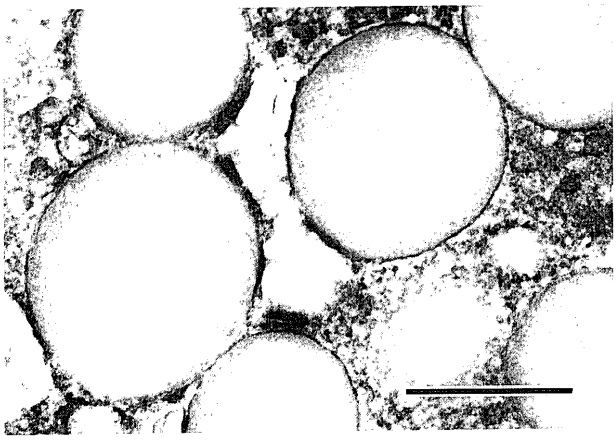

Fig. 2. Ultrastructural localization of GLUT4 introduced into a pancreatic acinar cell. Electron-dense deposit representing GLUT4 is localized along the membrane of zymogen secretory granules. Bar=0.5 μm.

was localized in the membrane of zymogen secretory granules stored in the supranuclear cytoplasm of the acinar cells (Fig. 2).

IV. Discussion

We have shown in this work that glucose transporters were successfully expressed in pancreatic acinar cells *in situ* by the *in-vivo* electroporation method. Immunohistochemical examination revealed that each isoform of glucose transporters was localized to distinct membrane domains.

Pancreatic acinar cells are highly differentiated secretory epithelial cells. Most of the cell body is covered with the basolateral plasma membrane. Tiny apex is covered with the apical plasma membrane that directly faces the narrow central lumen of the acinus. GLUT1 was localized in the basolateral membrane domain of acinar cells. GLUT1 was found in the basolateral domain in the epithelial cells of uriniferous tubules and collecting ducts in the kidney [27, 35]. When introduced in MDCK cells and Caco-2 cells, it was at the basolateral domain [9, 13]. When introduced into the hepatocytes *in vivo*, it was restricted to the sinusoidal domain [17]. These observations show that GLUT1 is similarly targeted to the basolateral domain in the hepatocytes and pancreatic acinar cells *in situ* as well as in cultured epithelial cells, suggesting a common targeting mechanism to the basolateral domain.

GLUT3 and GLUT5 were restricted to the apical membrane domain in pancreatic acinar cells. Similar apical localization was observed in Caco-2 and MDCK cells [7, 9, 11, 13], where apical membrane proteins are considered to be directly transported to the apical membrane [6, 18, 37]. When introduced into hepatocytes *in situ*, they were present along the whole aspects of the plasma membrane with significant enrichment to the bile canalicular membrane [17]. The apparent differences between pancreatic acinar cells and hepatocytes may be attributed to the unique targeting mechanism in hepatocytes [36, 38]. Apical proteins such as GLUT3 and GLUT5 may be directly inserted to the apical membrane in pancreatic acinar cells as observed in MDCK cells, making a marked contrast to the indirect targeting of apical proteins in hepatocytes, where transcytosis from the basolateral membrane plays a major route of apical delivery.

The expressed GLUT4 was localized to the supranuclear cytoplasmic regions in pancreatic acinar cells. Nomarski images indicated that these GLUT4-positive regions coincided with those of accumulation of zymogen granules. Ultrastructural immunocytochemistry revealed that GLUT4 was localized at the limiting membrane of zymogen granules, showing that GLUT4 is targeted to the secretory granules. In adipocytes and muscle cells, most of GLUT4 is localized in the cytoplasmic vesicles and tubules closely associated with or incorporated in the endosomal system and the trans-Golgi network [14, 19, 20]. Insulin stimulation induces the exocytosis of these GLUT4-containing vesicles and tubules, thereby increasing the number of GLUT4 molecules on the cell surface. When insulin signal is turned off, GLUT4 on the plasma membrane is retrieved to the cytoplasmic compartments via endocytic pathway. Such translocation of GLUT4 comprises the major cellular mechanism in the increase of glucose transport activity in insulin-sensitive cells. When GLUT4 was expressed in CHO cells or MDCK cells, most of the expressed GLUT4 was retained in the vesicles and tubules in the cytoplasm [1, 13], suggesting that GLUT4 is targeted to the cytoplasmic vesicular compartments. When GLUT4 was expressed in the neuroendocrine cell line PC 12, it was targeted to the large dense-core vesicles of the regulated secretory pathway [8]. Moreover,

GLUT4 is endogenously expressed in hormone-secreting atrial cardiac myocytes, and electron microscopic examination clearly showed that GLUT4 is targeted to the limiting membrane of secretory granules in addition to the trans-Golgi network and tubulovesicular compartment [20]. In all cases examined, GLUT4 did not accumulate on the plasma membrane. Instead, it was sequestered to the cytoplasmic vesicular compartments. When regulated secretory system is present, GLUT4 is delivered to this pathway as shown in PC 12 cells, hormone-secreting cardiac myocytes, and the present pancreatic acinar cells. Regulation in the exocytosis of hormone and zymogen granules apparently does not have any physiological relationship to the insulin-regulated trafficking of GLUT4. However, accumulation of GLUT4 in the regulated secretory compartments may represent a possible relationship between the GLUT4 trafficking mechanism and the regulated secretion machinery. Regulated translocation mechanism may possibly be evolved from the regulated exocytosis of secretory granules and subsequent membrane retrieval from the plasma membrane.

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VI. References

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