Kor J Physiol (1991) 25(2), 115-123

Molecular Association of Glucose Transporter in the Plasma Membrane of Rat Adipocyte

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= ABSTRACT =

Molecular association of glucose transporters with the other proteins in the plasma membrane was assessed by gel electrophoresis and immunoblot techniques. Approximately 31.5±5.7% of GLUT-4, 64.8±2.7% of clathrin, 48.7% of total protein in the plasma membrane (PM) were found insoluble upon extraction with 1% Tx-100. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that the Tx-100 insoluble PM fraction contained about 4 major polypeptides with apparent molecular weight of above 200, 100-120, 80 and 30-35 KDa that were readily removed upon wash with a high pH buffer which is known to remove clathrin and 0.5 M Tris-buffer which is known to remove assembly proteins (AP).Immunoblotting of GLUT4 and clathrin against specific antibodies showed that GLUT-4 and clathrin were co-solubilized up to 84.6% and 82.7% respectively by wash with a high pH buffer and 1% Tx-100. When the membrane was pre-washed with a high pH buffer and 0.5 M Tris solution,GLUT4 and clathrin were not solubilized further suggesting that GLUT4 molecules are in molecular association with clathrin,AP and/or other extrinsic membrane proteins in plasma membrane and the formation of clathrin-coated structures might be involved in insulin stimulated glucose transporter translocation mechanism.

Key Words: Glucose transporter, Glucose transporter translocation, GLUT4, AP, Immunoblotting, Silver stain.

INTRODUCTION

Stimulation of glucose transport by insulin in muscle and adipose tissues represents one of the most important, short-term control mechanism for maintenance of blood glucose level in vertebrates. Although detailed molecular events are not understood, it is generally accepted that insulin stimulates glucose transport in adipocytes largely by redistributing more glucose transporters from an intracellular storage compartment to the plasma membrane (Cushman & Wardzala, 1980; Suzuki & Kono, 1980). Many important membrane-bound enzymes and receptors are now known to distribute themselves between intracellular organelles and the plasma membrane utilizing specific membrane traffic pathways involving endocytosis (Gibbs et al, 1986; Corvera, 1990), and it is likely that the insulin-mediated glucose transporter redistribution utilizes a similar pathway.

Insulin increases the glucose transporter content in PM by more than 10 fold with a concomitant reduction in glucose transporter content in the intracellular storage pool (Ezaki et al, 1986; Calderhead et al,

Received Sept 8, 1991; Accepted Dec 18, 1991.

This work was supported in part by a GENETIC ENGINEERING RESEARCH GRANT from the MINISTRY OF EDUCATION(1991)

1990; Harrison et al, 1990). The insulin-induced glucose transporter recruitment to PM is very fast with an estimated half time of less than 5 min (Saltis et al, 1991). Upon termination of insulin treatment, net movement of glucose transporters from PM to the intracellular storage pool occurs, re-establishing the basal steady-state distribution. This net internalization of glucose transporter from PM to the storage vesicles upon insulin removal is also fast, showing a half-time indistinguishable from that of recruitment (Karnieli et al, 1981). Furthermore, the intracellular storage vesicles of glucose transporters in adipocytes have been isolated by immunoadsorption, where glucose transporters are 100~300 times more concentrated than in PM (James & Pilch, 1988; Zorzano et al, 1989). This preparation represents a small (2~5% in terms of protein) subpopulations of low density microsomes of adipocytes known as insulin-regulatable glucose transporter vesicles (IRGTV). IRGTV contains GLUT4, the major transporter isoform in adipocytes, and devoid of GLUT1, a minor isoform found in adipocytes.

The origin of IRGTV can be considered by two models. In one model, GLUT4 is sorted to IRGTV via a signal mediated process at trans-Golgi network as a regulated secretory proteins are sorted (Blok et al., 1988), and when insulin is added, this triggers the exocytosis of IRGTV by a mechanism analogous to the regulated secretion. GLUT1 are targeted directly to the plasma membrane via a default pathway, and then only GLUT4 enters into IRGTV from the plasma membrane via a specific endocytotic pathway similar to the receptor-mediated endocytosis (Stoorvogel et al, 1987; Lund et al, 1990). In this model, GLUT4 constantly recycles between the intracellular pool and the plasma membrane and insulin shifts the steady-state distribution of GLUT4 between the two pools. Intracellular vesicles containing GLUT1 would represent an intermediate of the default pathway. The first model does not explain the rapid, net return of glucose transporter that occurs upon insulin removal discussed above. Nonspecific fluid-phase endocytosis is too slow to account for this rapid internalization (Brown et al, 1988). The second model, on the other hand, readily accounts for the high transporter concentration in IRGTV and the rapid transporter internalization.

Therefore, this study was designed to test the second model discussed above by characterization of possible molecular association of glucose transporter with extrinsic proteins such as clathrin, assembly protein (AP-2) or certain proteins.

METHOD

Adipocyte isolation

Male rats (Sprague Dawley, 170~200 g) were used throughout. The rats were anesthetized with CO_2 and killed by decapitation between 8 and 9 a.m. and the epididymal fat pads were removed and rinsed in buffer. The buffer employed for all incubations was a Hepes-bufferd Krebs-Ringer solution (KRH) containing 130mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 2.5 mM NaH_2PO_4, 2.5 mM $CaCl_2$, and 10 mM Hepes adjusted to pH 7.4 with 10N NaOH. Adipocytes were isolated by a modification of the method of Rodbell (1964). The tissue was minced, placed in a polypropylene vial containing KRH with 0.5 mg/ml collagenase, 2% bovine serum albumin, and 2 mM D-glucose, and shaken for 30 min at 37°C. The cell suspension was then filtered through a silk mesh, the infranatant was withdrawn, and the cells were washed 4 times with KRH containing 1% bovine serum albumin and 1 mM D-glucose. The adipocyte suspension was adjusted to 40% packed cell volume and maintained at 37° C without shaking for a 45 min stabiliza-

tion period.

Preparation of subcellular membrane fractions

The adipocytes were washed once with TES homogenization buffer containing 20 mM Tris, 1 mM EDTA, and 8.7% sucrose that had been equilibrated to 18°C. The washing buffer was removed, and fresh TES was added. The tubes were immediately transferred into a cold room (4°C), and the cells were homogenized with a 50ml Potter-Elvehjem grinder (Thomas Scientific Co., specific clearance of 0.15 mm), which had been previously equilibrated to 4°C. Preparation of subcellular membrane fraction was done by differential centrifugation according to Simpson et al. (1983) method. Homogenates were centrifuged at 12,000rpm (JA-20) for 15min. The pellet was resuspended in TES buffer and homogenized in 2ml homogenizer with 20 strokes. The homogenate was applied on 38.5% sucrose cushion and centrifuged at 23,000 rpm (SW-27) for 1hr. After centrifugation, the interface layer plasma membrane was collected and pelleted in TES buffer under the same centrifugal force. The pellet was resuspended in a minimal volume of TES buffer and stored at −70° C until use. On the other hand, the supernatant of 12,000 rpm (JA-20) was centrifuged at 48,000 rpm (Beckman, 75Ti) for 1 hr and pellet containing the low density microsomes (LDM) was resuspended in a minimal volume of TES buffer and stored at −70°C until use.

Gel electrophoresis and immunoblotting

Electrophoresis was performed according to Laemmli (1970) with various sample preparations. Proteins were transferred to nitrocellulose paper in buffer consisting of 20% methanol, 380 mM glycine and 25 mM Tris, pH 8.3 for 4 hours with 100 volts. Following transfer, the paper was stained with 10% Ponceaus S solution and the gel was stained with Coomassie blue to confirm the electroblot. After check the transfer with Ponceaus S stain, the color was removed by washing with Tris-buffered saline (TBS) containing 150 mM NaCl and 10 mM Tris, pH 7.4. The filter papers were blocked with 5% non-fat dry milk in TBS for 1 hr at 22°C in 1% non-fat dry milk TTBS (TBS plus 0.05% Tween 20), and then reacted with ^{125}I-protein A (0.1 μCi/ml, Amersham). Dried blots were autoradiographed at −70°C with intensifying screen. The intensity of the autoradiographed film was quantified using Gilford UV-VIS spectrophotometer (Response series TM). Rabbit anti-GLUT4 polyclonal sera were raised against two synthetic polypeptides corresponding to the C-terminus amino acid sequence of 443-483 or N-terminus amino acid sequence of 1-12 of the insulin-regulatable rat glucose transporter or GLUT4 (James et al, 1989). Both GLUT4 and GLUT1 antisera were used at 1:300 dilution and rabbit antisera against light chain of brain clathrin were used at 1:200 dilution. On the other hand, the gel was visualized by silver staining according to Morrissey (1981) method. The procedure was as follows.

1) Placed gel in 50% methanol + 0.1% formaldehyde in 100 ml

2) Shook gel overnight

3) Moved gel in 500 ml of 0.5 mg% dithreitol (DTT) for 30 min.

4) Moved gel in 250 ml of 0.1% $AgNO_3$ for 30 min.

5) Rinsed gel quickly once with distilled water and twice with developer solution containing 3% Na_2CO_3 + 0.5% formaldehyde

6) Placed gel in developer until color reached an apropriate intensity

7) Added 0.1 volume of 2.3M citric acid and agitated for 10 min.

8) Washed gel for 30 min with distilled water 2~3 times.

9) Stored gel in 0.03% Na_2CO_3 at room

temperature.

The intensity of the colored bands was quantified using a LKB ultrascan XL laser densitometer and Hoefer dual speed scanning densitometer (GS 300).

Protein analysis

The total protein was measured essentially by the method of Lowry et al. (1951).

RESULTS

Polypeptide-profile of the adipocyte plasma membranes

Gel electrophoresis of adipocyte plasma membrane shows that there are about 30 or more polypeptides in intact membrane (Fig. 1 & Fig. 2A). Among them, 4 major polypeptides with apparent molecular weight of 180-200, 110, 80 and 30-33 K dalton (KDa) are readily removed by washing with a high pH buffer (pH 11.0), 0.5M Tris-HCl and 1% Tx-100. Treatment of 1% Tx-100 alone removed somewhat above 180, 100-110, and 80 KDa polypeptides which are considered to be intrinsic proteins (Fig. 2B). When the membrane was prewashed with a high pH buffer and washed with 1% Tx-100, polypeptides of 180-200, 120-160, 100-110, 80, 30-35KDa were removed partially (Fig. 2C). When the membrane was prewashed with a high pH buffer and 0.5M Tris, 100-120 KDa polypeptides were removed predominently (Fig. 2D).

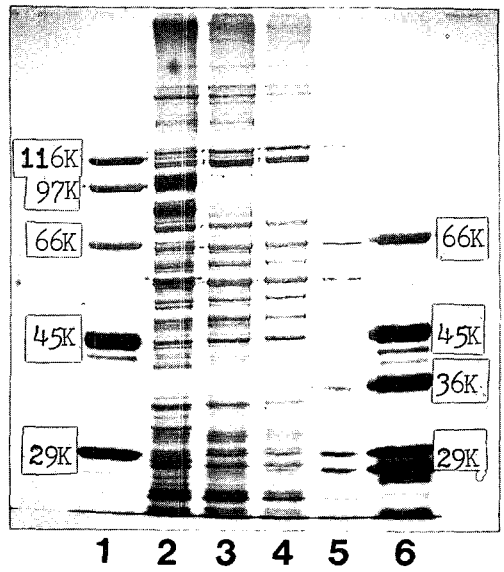

Fig. 1. Silver stained gel electrophoretic profile of the adipocyte plasma membranes treated with 1 % Tx-100, high pH buffer and 0.5 M Tris. Lane 1 contains a high molecular marker from Sigma Chemical Co. Lane 2 contains intact plasma membrane. Lane 3 contains 1 % Tx-100 insoluble proteins. Lane 4 contains 1 % Tx-100 insoluble proteins prewashed with a high pH buffer. Lane 5 contains 1 % Tx-100 insoluble proteins prewashed with a high pH buffer and 0.5 M Tris. Lane 6 contains a low molecular marker from Sigma Chemical Co.

Effect of high pH,Tris and Tx-100 on the immunoblot of GLUT4 of the plasma membrane

The effect of treatments of high pH, Tris and Tx-100 on the solubilization of GLUT4 were determined by quantitative immunoblotting with specific antibodies for the C-terminal amino acid, N-terminal amino acid and insulin-regulatable glucose transporter (1F8). The immunoblots of GLUT4 in the adipocyte plasma membrane treated with 1% Tx-100, high pH+1% Tx-100, and high pH+0.5M Tris+1% Tx-100 appeared in Fig. 3A. The scanning analysis of immunoblots shows that GLUT4 polypeptides were dissociated with the above treatments by 68.3, 83.6, and 86.3% of the initial (Fig. 3B).

Effect of high pH, Tris and Tx-100 on the amount of total protein, GLUT4 and clathrin of the plasma membrane

Table 1 shows that 1% Tx-100 removed

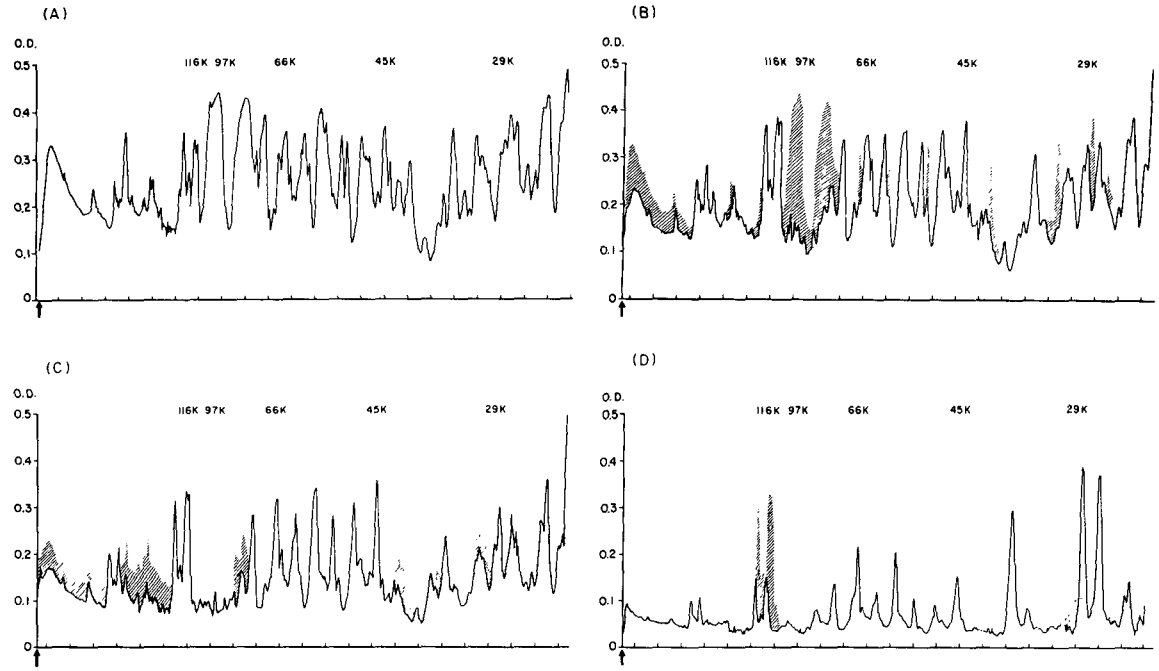

Fig. 2. Densitometric scanning profile of the silver stained gels showed in Figure 1. For scanning, LKB ultrascan XL laser densitometer was used. (A)(B)(C)&(D) correspond to Lane 2,3,4&5 of Figure 1.

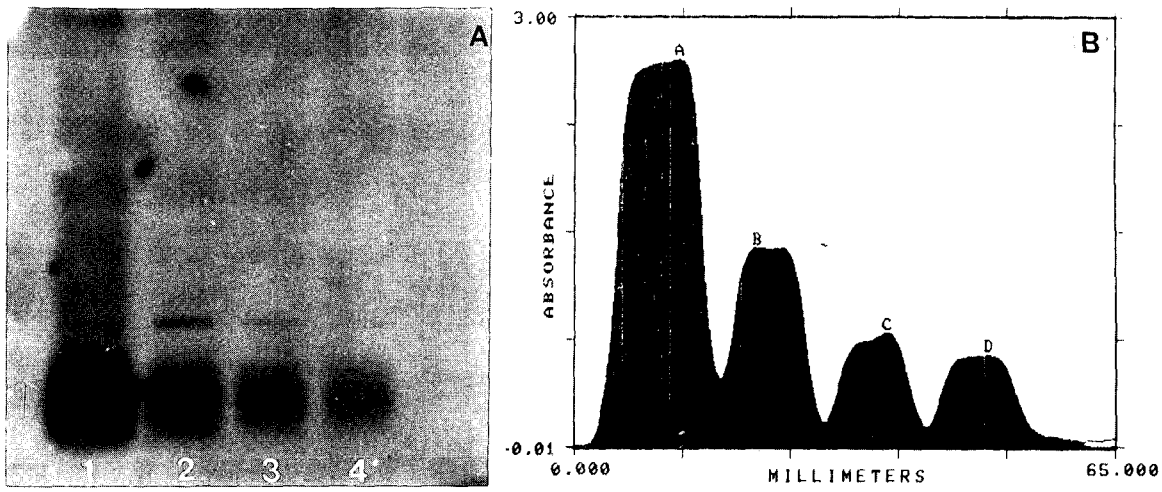

Fig. 3A. Effect of high pH, Tris and Tx-100 on the immunoblot of GLUT4 of the plasma membrane. A specific antibody for the C-terminal amino acid was used in this Figure. Lane 1 contains GLUT4 of intact plasma membrane. Lane 2 contains GLUT4 of 1 % Tx-100 insoluble plasma membrane. Lane 3 contains GLUT4 0f Tx-100 insoluble plasma membrane prewashed with a high pH buffer. Lane 4 contains GLUT4 of Tx-100 insoluble plasma membrane prewashed with a high pH buffer and 0.5 M Tris.

Fig. 3B. Quantitation of the immunoblots showed in 3A. For scanning, Gilford Response spectrophotometer was used. Peaks A,B,C & D represent the amounts of GLUT4 in various preparations.

Table 1. Effect of high PH, Tris and Tx-100 on the amount of total protein, GLUT4 and Clathrin of the PM

Treat	Amount		
	GLUT4 (%)	Clathrin (%)	Total protein (%)
Without	100	100	100
Tx-100	31.7±5.7	64.8±2.7	48.7
Alkali+ Tx-100	16.4±3.8	17.3±2.5	27.6
Alkali+ Tris-Tx-100	13.7±4.1	13.2±3.2	16.7

The concentrations of Tx-100 and Tris were 1% and 0.5M respectively.
Amount represents the amount of protein remained after each treatment.
Each value denotes mean±S.E. except total protein.

31.7±5.7% of GLUT4, 64.8±2.7% of clathrin and 48.7% of total protein. When the membrane was prewashed with a high pH buffer and treated with 1% Tx-100, the polypeptides were stripped prominently by 16.4±3.8% of GLUT4, 17.3±2.5% of clathrin and 27.6% of total protein. When the membrane was prewashed sequentially with a high pH buffer and 0.5 M Tris, and then treated with 1% Tx-100, the polypeptides were more removed by 13.7±4.1% of GLUT4, 13.2±3.2% of clathrin and 16.7% of total protein. These results implicate that GLUT4 and clathrin may be co-localized in the plasma membrane of adipocyte.

Effect of various concentrations of Tx-100 on the GLUT4 extraction of the plasma membrane

The effect of different concentrations of Tx-100 on the solubilization of GLUT4 was measured by immunoblotting of C-terminal peptides. No significant changes in the amount of GLUT4 of the plasma membranes treated with 0.5~3% Tx-100 were detected (Fig. 4).

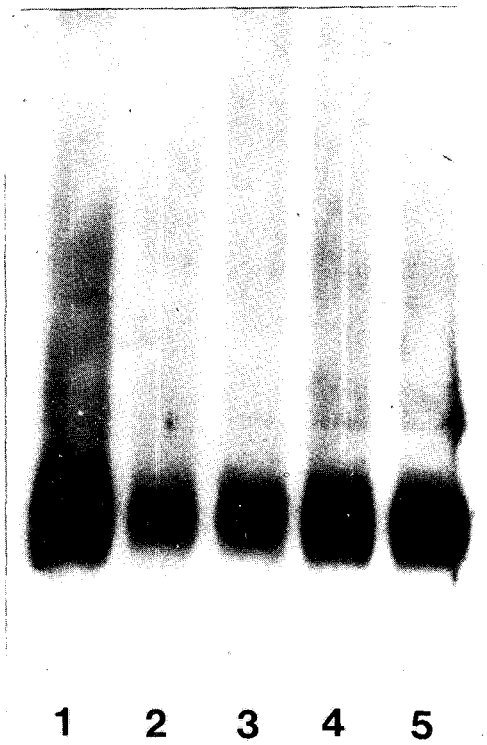

Fig. 4. Effect of various concentrations of Tx-100 on the GLUT4 extraction of the plasma membrane. Lanes 2, 3, 4 & 5 contain GLUT4s of plasma membranes treated with 0.5, 1, 2 & 3 % Tx-100.

DISCUSSION

It has been known for many years that insulin stimulates the rate of glucose transport in specific target tissues. Both the insulin receptor (Ebina et al, 1985) and the glucose transporter (Mueckler et al, 1985) are integral proteins of the membrane. Although the signalling event between the two proteins is not known, it has been proposed that stimuation of transport activity by insulin may occur by

recruitment of transporters to the cell surface (Cushman & Wardzala, 1980; Suzuki & Kono, 1980). Lienhard (1983) proposed that the transporter moves by exocytotic insertion and endocytotic retrieval, and insulin stimulates fluid-phase endocytosis in insulin-sensitive cell. However, the machinery for the endocytosis and retroendocytosis is not known well. One could speculate that glucose transporter molecules can be internalized via coated pits in which the protein concentration occurred by direct molecular interaction with a specific membrane protein or proteins. The coated pit is assembled from two heteromolecular subunits: The triskelion and the AP subunit (Mahaffey et al, 1990). Each triskelion is a trimeric structure composed of three clathrin heavy chain molecules (180KDa) and three clathrin light chain molecules (33-35KDa). This subunit is the building block for the clathrin polygonal lattice (Pearse & Crowther, 1987). Intercalated between the lattice and the plasma membrane is the AP complex consisting of two copies each of three different molecules with molecular weight of 100~116, 46~648 and 14~17 KDa (Heuser & Keen, 1988).

In this experiment, a possible molecular association of GLUT4 with clathrin, assembly proteins (AP) and other proteins was examined. Gel electrophoresis of adipocyte membrane shows that more than 30 polypeptides exist in intact membrane (Fig. 1). When the membrane was treated with 1% Tx-100, the polypeptides of above 200, 100 ~120, 80, 35 and 33KDa were removed, indicating that rest of them are extrinsic membrane proteins (Fig. 2B). When the membrane was prewashed with a high pH buffer, some extrinsic proteins of 180~200, 120~160, 80, 40, 35 and 33KDa were further solubilized (Fig. 2C). Treatment of a high salt solution (0.5M Tris) removed most of extrinsic proteins including 100~116, 46~48 and 14~17KDa (Fig. 3D). These results are agreed with Mahaffey et al (1990) showing that an alkaline pH removes clathrin and a high salt buffer removes the AP-2 and the triskelion subunits.

On the other hand, GLUT4 molecules in plasma membrane were also affected by treatment with a high pH, a high salt and Tx-100 solution. The quantitative analysis of immunoblotting of GLUT4 shows that the amounts of GLUT4 were decreased by 68.3% with 1% Tx-100 implicating that GLUT4 molecules are associated with some extrinsic proteins and co-sedimented (Mueckler et al, 1985). When the membrane was prewashed with a high pH buffer or high pH buffer and a high salt solution, the immunoblots were more decreased by 83.6% or 86.3% of the total (Fig. 3). The immunoblotting of clathrin light chain shows that the clathrin molecules were dissociated with a high pH buffer by above 85% of total, but little affected by a high salt treatment (Table 1). The amount of total protein was decreased by 48.7% with 1% Tx-100 solution. However, the amount of total protein was readily decreased when the protein was prewashed with a high pH buffer or a high pH buffer and a high salt solution (Table 1).

From the above results it is suggested that GLUT4 molecules might be associated with clathrin and AP molecules in the membranes, and the formation of clathrin-coated structures might be directly involved in insulin stimulated glucose transporter translocation mechanism.

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