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GLUT Phosphorylation May be Required to GLUT Translocation Mechanism

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In this work, GLUTs phosphorylations by a downstream effector of PI3-kinase, PKC-ζ, were studied, and GLUT4 phosphorylation was compared with GLUT2 phosphorylation in relation to the translocation mechanism. Prior to phosphorylation experiment, PKC-ζ kinase activity was determined as 20.76±4.09 pmoles Pi/min/25 ng enzymes. GLUT4 was phosphorylated by PKC-ζ and the phosphorylation was increased on the vesicles immunoadsorpted from LDM and on GLUT4 immunoprecipitated from GLUT4-contianing vesicles of adipocytes treated with insulin. However, GLUT2 in hepatocytes was neither phosphorylated by PKC-ζ nor changed in response to insulin treatment. It was confirmed by measuring the subcellular distribution of GLUT2 based on GLUT2 immunoblot density among the four membrane fractions before and after insulin treatment. Total GLUT2 distributions at PM, LYSO, HDM and LDM were 37.7±12.0%, 42.4±12.1%, 19.2±5.0% and 0.7±1.2% in the absence of insulin. Total GLUT2 distribution in the presence of insulin was almost same as that in the absence of insulin. Present data with previous findings suggest that GLUT4 translocation may be attributed to GLUT4 phosphorylation by PKC-ζ but GLUT2 does not translocate because GLUT2 is not phosphorylated by the kinase. Therefore, GLUT phosphorylation may be required in GLUT translocation mechanism.

Key Words: GLUT2, GLUT4, Phosphorylation, PKC-ζ, Translocation, Immunoadsorption, Immunoprecipitation, Adipocyte, Hepatocyte

INTRODUCTION

Many of the metabolic effects of insulin are mediated by dephosphorylation of rate-limiting enzymes in various metabolic pathways (Pugazhenthi & Khandelwal, 1995; Villar-Palasi & Guinovart, 1997). On the other hand, insulin stimulates the phosphorylation of a number of proteins in fat cells (Tanti et al, 1994; Standaert et al, 1999; Walaas et al, 1999; Hresko et al, 2000). Several protein kinases have been shown to be activated when cells are incubated with insulin (Bandyopadhyay et al, 1999; Katani et al, 1999; Standaert et al, 1999) although the function of these insulin-stimulated phosphorylation reactions has not been established. We found recently that insulin responsive glucose transporter, GLUT4, was phosphorylated directly by protein kinase C-zeta, a downstream effector of phosphatidylinositol 3-kinase (PI3-kinase) (Hah, 2000 in press) and the phosphorylation increased in GLUT4-containing vesicles purified from insulin treated cells implying that the transporter phosphorylation might be required in GLUT4 translocation mechanism in adipocyte (Piper et al, 1993). However, so far no report on a glucose transporter of hepatocyte, GLUT2, phosphorylation by PKC-ζ has been published because GLUT2 has not been a part of interest in insulin signal transdcution study for the reason that insulin does not trigger GLUT2 translocation in the hepatocytes. At this point, questions arise how insulin induces GLUT4 translocation but does not induce GLUT2 translocation in spite of that insulin phosphorylates insulin receptors as upstream of insulin signaling system of both adipocytes and hepatocytes (White, 1997; Rother et al, 1998), and how the difference of insulin sensitivity between in-

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sulin responsive cells such as adipose cell and muscle cell, and insulin unresponsive cells such as liver cell and blood cell, occurs. There are evidences that PI3-kinase is a crucial enzyme in the signal transduction of insulin in the glucose metabolism of the liver and peripheral tissue (Kahn, 1994; Shepherd et al, 1996; Daniel et al, 1999) suggesting a role of PKC-ζ, a downstream effector of PI3-kinase, in the control of glucose transport and phosphorylation in hepatocytes. We hypothesize that insulin sensitivity is attributed to glucose transporter translocation mechanism in which the transporter phosphorylation may contribute a crucial role for the translocation. Therefore, in the present study, we tested whether PKC-ζ directly phosphorylate GLUT2 in the hepatocytes as same as GLUT4 in the adipocytes. The effects of insulin on the phosphorylation were also examined in order to compare its effects on insulin responsive cell, adipocyte, with insulin unresponsive cell, hepatocyte.

METHODS

Materials

Collagenase (C-5138), insulin (porcine crystalline) and bovine serum albumin were purchased from Sigma (St. Louis, MO). 1F8 and GLUT2 monoclonal antibodies were purchased from Biogenesis (Brentwood, NH). Anti-calnexin CT (polyclonal antibody) was from Stress Gen Biothechnologies Inc (Victoria, BC, Canada). IP_3R-3 monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). PKC-ζ, Akt, PKC-ζ-pseudosubstrate, Mg^{2+}/ATP and ADB II were from Upstate Biotechnology (Lake Placid, NY). PS and PIP_3 were from Matreya Inc. (Pleasant Gap, PA). (γ-^{32}P) ATP was from NEN Life Science Products (Boston, MA). ECL Western Blotting detection reagent was from Amersham Pharmacia Biotech (Buckinghamshire, England). Horseradish-peroxidase (HRP)-protein A was from Zymed (San Francisco, CA). Reacti-gel (GF-2000) was purchased from Pierce (Rockford, IO). All other reagents were from sources stated below and were of reagent grade.

Isolation of hepatocytes

Rats (Sprague-Dawley, male, 150~200 g body weight), were anesthetized with sodium pentobarbital (40 mg/kg) by intramuscular (IM) injection and subjected to liver perfusion (Seglen, 1973). The liver was first perfused 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, followed by perfusion with 100 ml of Hank's buffer containing $CaCl_2$ (2 mM) and collagenase (100 mg/150 ml). The liver was removed and transferred into a petri dish containing 50 ml of the collagenase buffer. After incubation at 37°C for 10 min, cells were filtered through a 60 μm nylon mesh and stood for 20 min at 4°C to get rid of RBC, which was aspirated out, and washed 3 times with cold Hank's working solution by centrifugation at 200 g (SS-34 rotor, 1,000 rpm). The resulting isolated cells were resuspended in Krebs-Ringer phosphate buffer containing 2 mM pyruvate and 1% BSA, with readjustment at pH 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 $1\sim2\times10^6$ cells/ml. Hepatocytes were counted by a Coulter electronic particle counter. One rat liver typically gave $10\sim12\times10^6$ isolated hepatocytes or 3~4 ml of packed cells.

Subcellular fractionation of hepatocyte

The procedure was essentially that of Fleischer & Kervina (1974) except homogenization method. The hepatocytes were slightly packed by centrifugation at 2,000 rpm (SS-34 rotor) for 3 min and made 50 % cell suspension in homogenizing solution containing 0.25 M sucrose and 10 mM HEPES at pH 7.4. The cell suspension was aliquoted by 1 ml in eppendorf tube and disrupted the cells by syringe method. In brief, the cells were passed 15 times through 22 gauge needle with 1 ml tuberculin syringe, and each homogenate was collected into 40 ml centrifuge tube. Whole homogenate was centrifuged at 200g (SS-34 rotor, 1,000 rpm) for 3 min, and the supernatant was saved and the precipitated unbroken cells were disrupted by the same method. This procedure was repeated three or four times and the collected supernatant was subfractionated by differential centrifugation. The whole supernatant was centrifuged at 1,450 g (SS-34 rotor, 3,500 rpm) for 10 min, and supernatant and pellet were obtained. The resultant pellet is known to contain plasma membrane, nucleus, and mitochondria (PM/NM) (Fleischer & Kervina, 1974). The supernatant was further subfractionated by centrifugation at 25,000 g (SS-34 rotor, 14,500 rpm) for

10 min. The resulting pellet is known to contain abundant lysosomes (lysosome enriched fraction or LYSO). The supernatant was centrifuged again at 48,000 g (50.2 rotor, 23,000 rpm) for 30 min yielding a pellet of high density microsomal membranes (HDM). The supernatant was then centrifuged at 200,000 g for 180 min, yielding a pellet of low density microsomal membranes (LDM). Thus, our subcellular fractionation schemes are similar to that of Ciaraldi et al (1986), except that we did not discard any particulate fractions.

Isolation of adipocytes and purification of GLUT4-containing vesicles

Adipocytes were isolated from epididymal fat pads of male Sprague Dowley rat weighing 180~220 g as described (Rampal et al, 1995). Briefly, total membranes were fractionated to plasma membrane enriched fraction (PM), high density microsome (HDM) and low density microsome (LDM) by differential centrifugation (Simpson et al, 1983; Rampal et al, 1995). To get GLUT4-containing vesicles, protein A purified 1F8 antibody (James et al, 1988) as well as nonspecific normal mouse IgG were coupled each to Trisacryl beads (Reacti-Gel, GF-2000) at a concentration of 0.7 mg of antibody/ml of resin according to the manufacturer's instruction. The antibodies coupled on beads were quenched by 2 M Tris, pH 8.0, for 1 h at room temperature; incubated with 2% bovine serum albumin in phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for 2 h also at room temperature to block nonspecific binding; and washed five times with 1 ml of PBS. LDMs (200 g of protein) were then incubated with 50 μg of beads overnight at 4°C. The beads were settled down spontaneously in ice bucket, and unbound supernatants were collected for analysis. The beads bound GLUT4-containing vesicles were washed five times with 1 ml of PBS and twice with phosphorylation buffer at 4°C, and then the beads were used for phosphorylation experiment as described in the following paragraph.

In vitro phosphorylation of GLUT4-containing vesicles

A reaction mixture for phosphorylation consists of 50 μl of beads immunoadsorbed GLUT4-containing vesicles, 50 μl of phosphorylation buffer, 5 μl of PS (0.5 mg/ml), 5 μl of PIP_3, dipalmitoyl (0.05 mg/ml), 10 μl of human recombinant PKC-ζ (10 ng/μl), and [γ-^{32}P] ATP (50 μl, 3,000 Ci/mmol). In the case of PKB phosphorylation experiment, 20 μl of PKB-α recombinant protein expressed in Sf9 cells was added instead of PKC-ζ. The phosphorylation assay was started by adding [γ-^{32}P] ATP into the reaction mixture and incubated for 30 min at room temperature with vigorous shaking. Beads were then precipitated and washed two times with phosphorylation buffer. The immunoadsorbed materials were eluted with the same volume of double concentrated Laemmli (1970) sample buffer without 2-mercaptoethanol, which containing 0.125 M Tris-HCl, 4% sodium dodecylsulfate (SDS), 20% glycerol and appropriate amount of pyronin-X. The eluted solutions were then added by 1% mercaptoethanol and electrophoresed onto 10% acrylamide/glycine gel with 130 volts. Gels were transferred to nitrocellulose (NC) membrane with 105 volts for 1.5 h. The NC membranes were wrapped with Saran wrap and exposed overnight in a storage phosphor screen cassette, and phosphor images were quantitated in a PhosphorImager (Molecular Dynamics). Alternately, phosphorylated proteins were electrophoresed, and dried gels were used for phosphor image scanning in the PhosphorImager.

In vitro phosphorylation of GLUT2-containing subfractional proteins

A reaction mixture for phosphorylation of hepatocytes membranes consists of 13.9 μl (100 μg) of homogenates, 27.1 μl of phosphorylation buffer, 20 μl of PS (0.5 mg/ml), 20 μl of PIP_3-dipalmitol (0.05 mg/ml), 4 μl of $MgCl_2$ (750 mM), 5 μl of human recombinant PKC-ζ (10 ng/μl), and 10 μl of [γ-^{32}P]ATP (50 mM, 3,000 Ci/mmol). The phosphorylation assay was started by adding [γ-^{32}P]ATP into the reaction mixture and incubated for 30 min at room temperature with vigorous shaking. After incubation, 20 μl of 30% TCA were added and spun for 5 min at 14,000 rpm (Beckman Microfuge II). The precipitate was washed once with H_2O, resuspended in 25 μl of H_2O, added 25 μl of sample buffer (2X) without mercaptoethanol and 50 μl of sample buffer (1X) with mercaptoethanol. The next steps were similar to the above.

Gel electrophoresis and Western blotting

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) was performed according to the method of Laemmli (1970) with a slight modification as follows. Samples 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 transferred NC membrane was stained with 0.01% Ponceaus S and the molecular weight standards were marked. The membranes were then destained with TBS buffer (50 mM Tris-base, 150 mM NaCl) and blocked with 5% milk for 30 min at room temperature with shaking followed by incubation overnight with primary antibody in 1% milk/TBS. The next morning, the membranes were washed three times with TTBS (TBS+0.05% Tween 20) for 10 min each time 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 1min and exposed to X-ray film in the dark room. The X-ray films were developed at autodeveloper and images were quantitated by densitometer.

Protein measurement

Protein concentration was measured basically by Bradford (1976) dye binding assay (Bio-Rad).

RESULTS

Determination of PKC-ζ kinase activity

Prior to phosphorylation experiment by PKC-ζ, the enzyme kinase activity was determined. As shown in Fig. 1 the enzyme kinase activity was linear up to incubation for 20 min. In details, the enzyme kinase activities at incubation time for 0, 5, 10, 20 and 30 min were 149.37, 237.93, 400.17, 617.84 and 655.66 pmoles phosphate/25 ng enzyme, so the mean kinases activity of PKC-ζ was 20.76±4.09 pmoles phosphate/min/25 ng enzyme.

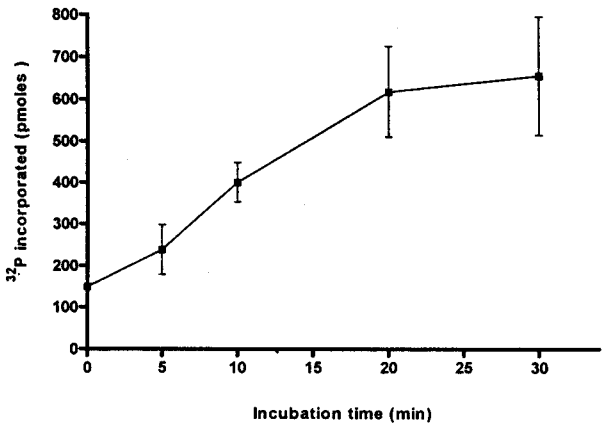

Fig. 1. PKC-ζ kinase activity. A reaction mixture consists of 10 μl of the substrate (ERMRPRKRQGSVRRRV), 20 μl of ADBII, 10 μl of lipid activator (0.5 mg/ml of PS and 0.05 mg of diglycerides), 2.5 μl of human recombinant protein kinase (10 ng/ μl), and 10 μl of [γ-^{32}P] ATP mixture (10 μl of [γ-^{32}P] 3,000 Ci/mmole+90 μl of $MgCl_2$/ATP cocktail). The mixture was incubated for 0, 5, 10, 20 and 30 min at 30°C and stopped reaction by adding 8.75 μl of 35% acetic acid, and then slowly transferred 30 μl of the mixture onto the center of a numbered P81 paper. The paper was washed by dipping 10 times into a large volume of 0.75% phosphoric acid and washed once more with acetone. The paper was transferred to a scintillation vial and added scintillation cocktail, and then counted. The enzyme kinase activity was calculated as liberated phosphate/min/25 ng enzyme.

Phosphorylation of GLUT4 in adipocytes by PKC-ζ

GLUT4-containing vesicles immunoadsorbed from LDM of rat adipocytes treated with and without insulin were phosphorylated as described in the methods. As shown in Fig. 2, GLUT4 was directly phosphorylated by PKC-ζ and the phosphorylation was affected by insulin. GLUT4 phosphorylation was increased at the vesicles immunoadsorbed from LDM of insulin treated adipocytes based on phosphoimage intensity and immunoblot with GLUT4 antibody, moreover GLUT4 phosphorylation was inhibited in the presence of PKC-ζ-pseudosubstrate (data not shown). In order to confirm that GLUT4 is phosphorylated by PKC-ζ, GLUT4 protein was immunoprecipitated with 1F8 antibody coupled with Trisacryl beads in the presence of 1% Triton X-100. Fig. 3 showed clearly that GLUT4 was phosphorylated by PKC-ζ. The phosphorylation also increased at GLUT4 immunoprecipitated from the GLUT4-contianing ves-

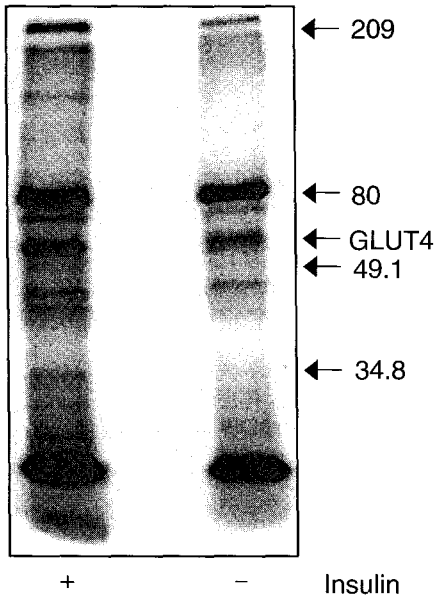

Fig. 2. Phosphoimages of GLUT4-containing vesicles phosphorylated by PKC-ζ. GLUT4-containing vesicles were purified from the rat adipocytes treated with and without insulin and phosphorylated by PKC-ζ as described in Methods. A reaction mixture consists of 50 μl of 1F8 beads, 100 μl of ADBII, 50 μl of PS (0.5 mg/ml), 20 μl of PIP_3 (0.05 mg/ml), 20 μl human recombinant PKC-ζ, and 50 μl of [γ-^{32}P]ATP mixture. The reaction mixture was incubated for 30 min at room temperature with vigorous shaking. After incubation the beads were washed with buffer and eluted proteins with Lammli sample buffer for 1 hr with vigorous shaking, and applied onto SDS/PAGE. The gels were transferred to NC membrane and analyzed by scanning phosphoimage of ^{32}P incorporated and immunoblotting with GLUT4 antibody.

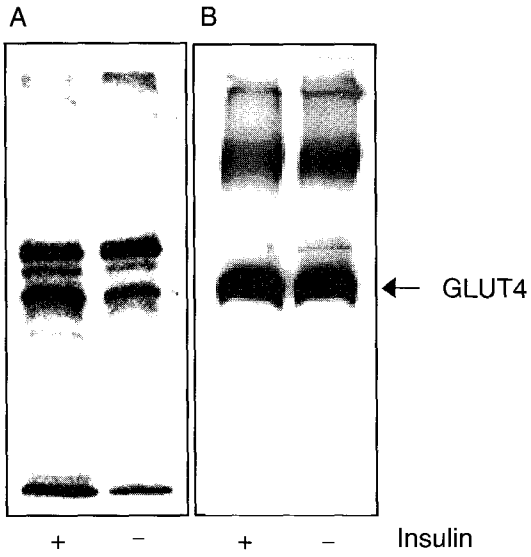

Fig. 3. Phosphoimages of the immunoprecipitated GLUT4 protein phosphorylated by PKC-ζ. GLUT4-containing vesicles were phosphorylated by PKC-ζ as described in Fig. 2 legend and solubilized in 1% Tx-100. GLUT4 immunoprecipitation was achieved by incubating 1F8 antibody coupled with Trisacryl beads. The beads were washed with buffer and eluted proteins with Lammli sample buffer for 1 hr with vigorous shaking. The proteins were applied onto SDS/PAGE and transferred to NC membrane and analyzed by scanning phosphoimage of ^{32}P incorporated and immunoblotting with GLUT4 antibody. A. Phosphoimage of GLUT4 immunoprecipitated. B. Immunoblot of GLUT4 immunoprecipitated with GLUT4 antibody.

icles treated with insulin.

Phosphorylation of GLUT2 in hepatocytes by PKC-ζ

GLUT2-containing PM, LYSO, HDM and LDM were treated with PKC-ζ in order to phosphorylate GLUT2 by the same way as GLUT4 phosphorylation. As shown in Fig. 4, GLUT2 was neither phosphorylated by PKC-ζ nor changed in response to insulin. In the present study, the subcellular distribution of GLUT2 among the four membrane fractions obtained from hepatocytes incubated either in the absence or presence of insulin was measured. Briefly, in the absence of insulin, total GLUT2 distributions at PM, LYSO, HDM and LDM were 37.7±12.0%, 42.4±12.1%, 19.2±5.0% and 0.7±1.2%. In the presence of insulin, total GLUT2 distributions were almost same as those in the absence of insulin within statistically permissible errors. These indicated that insulin did not induce GLUT2 translocation in the hepatocytes. However, the intensity of GLUT2 immunoblot were different from the total distribution. For example, relative intensities of GLUT2 immunoblots at PM, LYSO, HDM and LDM were 1 : 2 : 4 : 0.2 i.e. the intensity of GLUT2 at HDM was the highest implying that GLUT2 was concentrated at HDM. To determine more precisely whether or not PKC-ζ phosphorylate GLUT2, we attempted to purify GLUT2 protein by immunoadsorption and immunoprecipitation for further phosphorylation experiment. For the purification of GLUT2 protein by immunoprecipitation, GLUT2 antibody and protein A-beads were used to precipitate GLUT2 in the presence of 2% $C_{12}E_8$, but polyclonal GLUT2 antibody (Biogenesis, Brentwood) did not work on GLUT2 im-

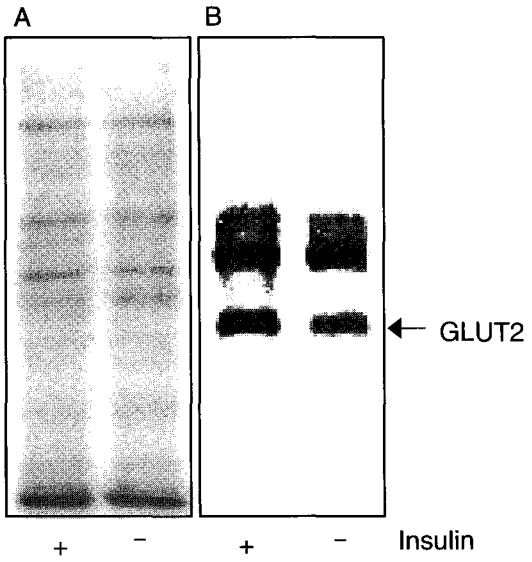

Fig. 4. Phosphoimages of GLUT2-containing HDM phosphorylated by PKC-ζ in hepatocytes. Hepatocytes were isolate, treated with and without insulin and fractionated to subcellular organelles such as PM, LYSO, HDM and LDM as described in Methods. Membrane proteins of HDM (100 μg) were phosphorylated in a reaction mixture consisting of 40 μl of homogenate, 20 μl of PS (0.5 mg/ml), 20 μl of PIP_3 (0.05 mg/ml), 4 μl of 750 mM $MgCl_2$, 5 μl of human recombinant PKC-ζ, and 10 μl of [γ-^{32}P]ATP mixture for 30 min at room temperature with vigorous shaking. The proteins were precipitated with 5% TCA and pellets were resuspended in H_2O, then applied onto SDS/PAGE. The gels were transferred to NC membrane and analyzed by scanning phosphoimage of ^{32}P incorporated and immunoblotting with GLUT2 antibody. A. Phosphoimage of HDM homogenate. B. Immunoblot of HDM homogenate with GLUT2 antibody.

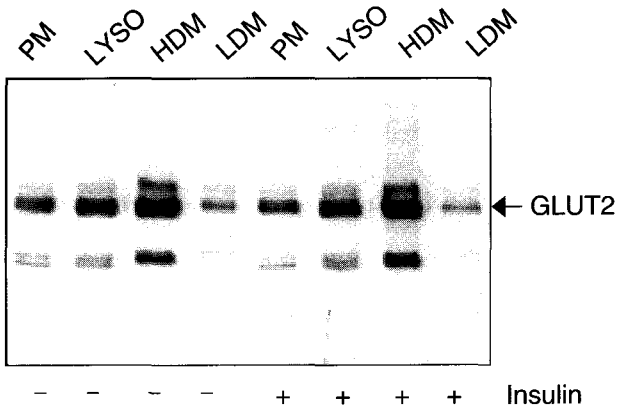

Fig. 5. Effect of insulin on the subcellular distribution in hepatocytes. Hepatocytes were isolated as discribed in Methods. Isolated hepatocytes were treated with and without insulin for 20 min at 37°C incubator and fractionated to subcellular organelles such as PM, LYSO, HDM and LDM. Each homogenate (30 μg) was applied onto SDS/PAGE and immunoblotted with GLUT2 antibody. The effect of insulin was determined by comparing the intensity of GLUT2 immunoblot of insulin treated group with that of insulin untreated group. Total GLUT2 distribution was calculated by intensity of GLUT2 immunoblot with GLUT2 antibody multiplied by total yield of each fraction.

munoprecipitation. Therefore GLUT2 immunoadsorption was tried using a ER marker such as calnexin (rabbit anti-calnexin C-terminus polyclonal antibody; Stress Gen Biotechnologies Corp, Victoria, Canada) and IP_3R-3 (Transduction Laboratories, Lexington) after HDM homogenate was tried to be phosphorylated by PKC-ζ because they are co-localized with GLUT2 protein at HDM as indicated above. Fig. 6 shows the immunoblot of GLUT2 and phosphoimage after being immunoadsorpted with calnexin antibody. As shown at left panel, GLUT2 protein seemed to be immunoadsorpted with calnexin antibody, however, phosphoimage was not scanned as shown at right panel. It represents that GLUT2 protein was not phosphorylated by PKC-ζ. Fig. 7 shows the immunoblot of GLUT2 and phosphoimage after being immunoadsorpted with IP_3R-3 antibody. As shown at left panel, GLUT2 protein seemed to be immunoadsorpted with IP_3R-3, however, phosphoimage was not appeared as shown at right panel. It again indicated that GLUT2 protein was not phosphorylated by PKC-ζ.

DISCUSSION

Insulin stimulates glucose transport in insulin responsive tissue cells such as adipocytes and muscle cells by increasing the translocation of the glucose transporter from an intracellular pool to the plasma membrane (Czech & Corvera, 1999; Olefsky, 1999; Pessin et al, 1999). In GLUT4 translocation mechanism, the protein phosphorylation is known to be an important step (Lawrence et al, 1990; Piper et al, 1993; Thorens et al, 1996) in which PI3-kinase activation is recognized to be required (Cheatham et al, 1994; Okada et al, 1994; Kotani et al, 1995; Yang et al, 1996; Czech & Corvera 1999). Recently PKC-ζ, one of downstream effectors of PI3-kinase, is

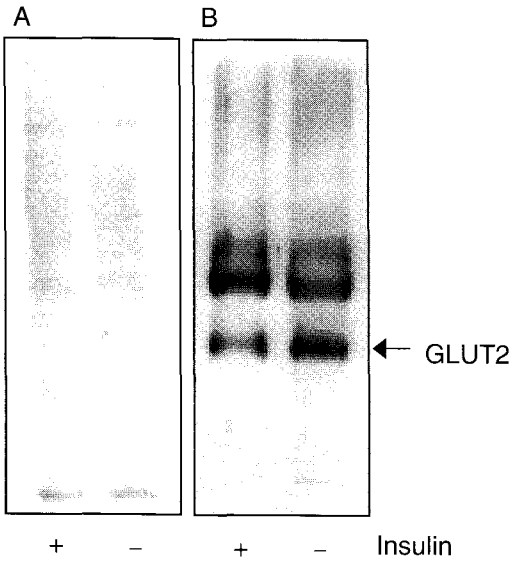

Fig. 6. Phosphoimages of GLUT2 immunoadsorpted with calnexin antibody. GLUT2 enriched HDM (100 μg) was sonicated in ice chilled bath and incubated with calnexin antibody overnight at cold room with rotation. The mixture was incubated with 50 μl of 50% agarose bound Protein A gel for 1 hr at cold room with rotation. The immunoadsorpted materials with 25 μl of agarose bound Protein A were phosphorylated in a reaction mixture consisting of 25 μl of agarose bound Protein A gel, 15 μl of buffer, 20 μl of PS (0.5 mg/ml), 20 μl of PIP_3 (0.05 mg/ml), 4 μl of 750 mM $MgCl_2$, 5 μl of human recombinant PKC-ζ, and 10 μl of [γ-^{32}P] ATP mixture for 30 min at room temperature with vigorous shaking. The gels were washed 3 times with PBS and eluted proteins with Lammli sample buffer, and applied onto SDS/PAGE. The gels were transferred to NC membrane and analyzed by scanning phosphoimage of ^{32}P incorporated and immunoblotting with GLUT2 antibody. A. Phsophoimage of GLUT2 immunoadsorpted with calnexin. B. Immunoblot of GLUT2 immunoadsorpted with GLUT2 antibody.

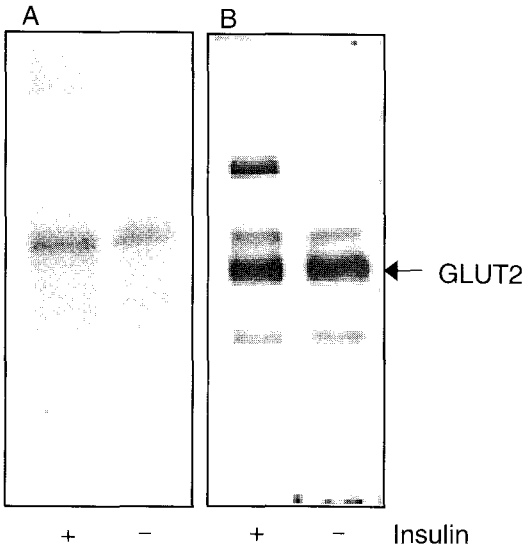

Fig. 7. Phosphoimages of GLUT2 immunoadsorpted with IP_3 antibody. HDM homogenate (100 μg) was incubated with 50 μl of IP_3 antibody coupled with Trisacryl beads overnight. The beads were separated and phosphorylated in a reaction mixture consisting of 50 μl of Trisacryl beads containing GLUT2, 20 μl of PS (0.5 mg/ml), 20 μl PIP_3 (0.05 mg/ml), 4 μl of 750 mM $MgCl_2$, 5 μl of human recombinant PKC-ζ, and 10 μl of [γ-^{32}P]ATP mixture for 30 min at room temperature with vigorous shaking. The beads were washed 3 times with PBS and eluted proteins with Lammli sample buffer, and applied onto SDS/PAGE. The gels were transferred to NC membrane and analyzed by scanning phosphoimage of ^{32}P incorporated and immunoblotting with GLUT2 antibody. A. Phosphoimage of GLUT2 immunoadsorpted with IP_3 B. Immunoblot of GLUT2 immunoadsorpted with GLUT2 antibody.

demonstrated to directly phosphorylate GLUT4 purified from LDM of rat adipocytes treated with and without insulin, and the GLUT4 phosphorylation increased in insulin treated adipocytes. This suggests that GLUT phosphorylation may contribute to insulin-induced GLUT translocation mechanism and determine insulin responsiveness between two distinct types of tissue cells such as adipocytes and hepatocytes in glucose transport system. So far no report on GLUT2 phosphorylation has been published. In the present study, two types of GLUT, GLUT2 and GLUT4, were attempted to be phosphorylated using PKC-ζ that is a well known protein kinase to phosphorylate glucose transporter (Standaert et al, 1997) and the GLUT phosphorylation susceptibility was evaluated with respect to GLUT translocation mechanism. Adipocytes and hepatocytes were isolated, treated with and without insulin, and subfractionated to PM, LYSO, HDM and LDM, respectively. GLUT4-containing vesicles of rat adipocyte LDM were purified and GLUT4 protein was further purified by immunoprecipitation for phosphorylation experiment by PKC-ζ (Kypriyanova & Kandror, 1999). In these preparations, PKC-ζ phosphorylates GLUT4 directly (Fig. 2, 3). GLUT4 phosphorylation was increased at the preparations obtained from adipocytes treated with insulin. These data are consistent with previous investigation (Hah 2000, in press) and opposite to

others (Gibbs et al, 1986; Joost et al, 1987; James et al, 1989). However it is still unclear whether the phosphorylation state of GLUT4 is involved in either signaling transporter translocation or triggering changes in transporter intrinsic activity (Lawrence et al, 1990; Reusch et al, 1991; Thorens et al, 1996). On the other hand, GLUT2 enriched HDM of rat hepatocytes was tried to be phosphorylated by PKC-ζ but GLUT2 was not phosphorylated as seen in phosphoimage scan (Fig. 4). To make sure that GLUT2 is not phosphorylated by PKC-ζ, GLUT2 protein was further purified by immunoadsorption and immunoprecipitation, and tried to get phosphorylation. Purification of GLUT2 protein by immunoprecipitation with GLUT2 antibody using protein A-beads in the presence of 2% $C_{12}E_8$ was tried, but GLUT2 immunoprecipitation was not successful. GLUT2 immunoprecipitation methods were modified several times by changing detergent as 1% Tx-100 instead of 2% $C_{12}E_8$, by changing bead as Trisacryl beads instead of protein A, and by changing protocol itself. However, GLUT2 immunoprecipitation by polyclonal GLUT2 antibody (Biogenesis, Brentwood) was failed. Therefore, purification of GLUT2 by immunoadsorption was performed using a ER marker such as calnexin and IP_3R-3 because ER is a major particulate in HDM and may co-exist with GLUT2 in the vesicular structure. Immunoadsorption of GLUT2 with these ER markers seemed to work. After phosphorylation of HDM protein by PKC-ζ, immunoadsorption of GLUT2 with calnexin and IP_3R-3 appeared on immunoblot of GLUT2 with GLUT2 antibody (Fig. 6, 7). Again GLUT2 phosphorylation by PKC-ζ was not affirmed on phosphoimage scan. It is very interesting that GLUT4 phosphorylation is induced by PKC-ζ and the phosphorylation increases in insulin stimulated state but GLUT2 phosphorylation is not increased by PKC-ζ, nor affected by insulin. In the present study, the subcellular distribution of GLUT2 among the four membrane fractions obtained from hepatocytes treated with and without insulin was measured. GLUT2 distributions at PM, LYSO, HDM and LDM were not changed before and after insulin treatment. These indicated that insulin did not induce GLUT2 translocation in the hepatocyte. These data are consistent with previous investigation (Hah et al, 1992). From the above result, it is clear that GLUT2 dose not translocate between subcellular fractions in hepatocyte upon insulin treatment due to no responsiveness to insulin. Taken together, present data suggest that GLUT4 translocation in adipocyte may be attributed to GLUT4 phosphorylation by PKC-ζ, a downstream effector of PI3-kinase but GLUT2 does not translocate because GLUT2 is not phosphorylated by the kinase, therefore, GLUT phosphorylation may be required in GLUT translocation mechanism.

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