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☐ Original Article ☐

Cloning and Expression of Rat Liver Type Glucose Transporter and Translocation by Insulin in Chinese Hamster Ovary Cells

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The 5'- and 3'-side half of liver type glucose transporter (GLUT2) cDNA was amplified from total RNA or mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR). The amplified 5'-side fragment of GLUT2 cDNA was inserted into pGEM4Z and named pGLGT1, and the 3'-side fragment of GLUT2 cDNA was inserted into the HindIII site of pGLGT1 to construct pGLGT2 which contains an entire open reading frame of GLUT2 cDNA. The GLUT2 cDNA in pGLGT2 was transferred to an eukaryotic expression vector (pMAM) to construct pMLGT, which was expressed in the insulin-sensitive Chinese hamster ovary (CHO) cells. Western blot analysis showed that the GLUT2 gene in pMLGT was expressed in the transfected CHO cells successfully. The GLUT2 content in the plasma membrane fraction of insulin-treated CHO cells expressing GLUT2 increased 3.8-fold compared to that of the control group. This result suggests that GLUT2, which is not subjected to translocation by insulin in the cells of its major distribution, can be translocated if it is expressed in the suitable cells sensitive to insulin action.

Key Words: Liver type glucose transporter (GLUT2), reverse transcriptase-polymerase chain reaction (RT-PCR), chinese hamster ovary (CHO) cells, insulin, translocation

Insulin lowers the blood glucose level by stimulating glucose entry into muscle and adipose cells (Schroer *et al.* 1986). The basic mechanism of this phenomenon is known to be due to the translocation of glucose transporters stored at low density microsomal fractions to plasma membranes by insulin treatment (Cushman and Wardzala, 1980) in these cells. However, this translocation principle applies only to the glucose transporter (GLUT4) present in muscle and adipocyte. Other types of glucose transporters with the exception of GLUT4 do not translocate themselves in response to insulin when they are present in the cells of their major distribution (Bell *et al.* 1990). Therefore, it raises a question about the possible role of insulin in the translocation of glucose transporters. It is assumed that the translocation of GLUT4 by insulin can be induced by two possible mechanisms. First, a specific amino acid sequence of the glucose transporter contains a signal responsible for translocation by insulin. Second, there may be tissue-specific signals playing a role in relation to insulin-related translocation signal pathways in specific cells that are responsive to insulin action.

To work out one of two possibilities, GLUT2 cDNA was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) and was subcloned into an eukaryotic expression vector. The expression of GLUT2 gene

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in Chinese hamster ovary (CHO) cells was analyzed by Western blot. The results in this paper suggest that GLUT2 can be redistributed to plasma membrane fraction in response to insulin if it is expressed in insulin-sensitive cells, as in the case of GLUT4 in muscle cells or adipocytes.

MATERIALS AND METHODS

Plasmids

A prokaryotic cloning vector (pGEM4Z) and an eukaryotic expression vector (pMAM) were purchased from Promega and from Clonetech, respectively. pECE-IR containing human insulin receptor cDNA was kindly provided by Dr. Rutter of the University of California in San Francisco.

Oligonucleotides

Oligonucleotides used to amplify GLUT2 cDNA by PCR were synthesized according to the sequence of GLUT2 cDNA (Fukumoto *et al.* 1988). They were as follows: primer 1, TGAGACAACAACTCCGCACACAACA (−24 ~1, sense); primer 2, TCCCAGCGACATGAAGACGGC (1152~1132, antisense); primer

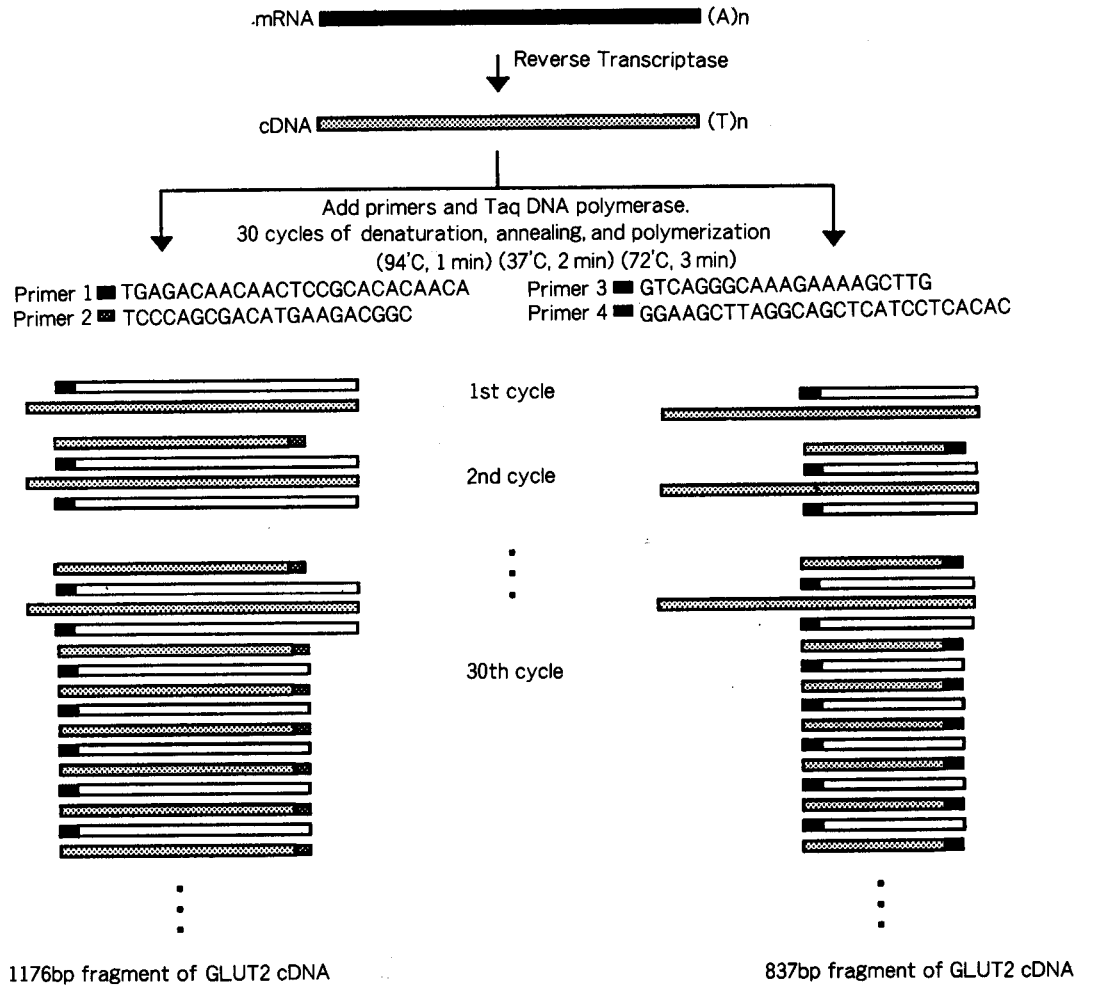

Fig. 1. *Strategy for the amtlification of GLUT2 cDNA using RT-PCR.*

3, GTCAGGGCAAAGAAAAGCTTG (753~773, sense); primer 4, GGAAGCTTAGGCAGCTCA-TCCTCACAC (1590~1564, antisense).

Amplification of GLUT2 cDNA by RT-PCR

Total RNA and mRNA were fractionated from male Sprague-Dawley rat (~150 g) liver according to the method of Sambrook *et al.* (1989). The complementary DNAs for these RNAs were synthesized by reverse transcriptase using oligo (dT) as a primer. Of the resulting cDNAs, 5'- or 3'-side fragments of GLUT2 cDNA were amplified by PCR in the presence of primers 1 and 2 or primers 3 and 4. The overall amplification strategy is shown in Fig. 1.

Cloning of GLUT2 cDNA

The amplified 5'-side fragment of GLUT2 cDNA was digested with *Hin*dIII and were purified with Geneclean kit (BIO101) after agarose gel electrophoresis. It was inserted into pGEM4Z digested with *Hin*cII and *Hin*dIII to construct pGLGT1. The amplified 3'-side fragment of GLUT2 cDNA was digested with *Hin*dIII and inserted into *Hin* dIII site (the end of 5'-half fragment of GLUT2) of pGLGT1 to construct pGLGT2, which contains an entire open reading frame of GLUT2 cDNA. The GLUT2 cDNA containing an entire open reading frame was excised out from pGLUT2 with *Xba*I and *Hin* dIII and pMAM was digested with *Sal*I. Their cohesive ends were filled with nucleotides by Klenow fragment. The two DNAs were ligated by T4 DNA ligase and the resulting recombinant DNAs were used to transform *Escherichia coli* DH5*α*. Of the recombinant plasmid DNAs isolated from the transformants, a recombinat DNA in which GLUT2 cDNA was inserted next to the long terminal repeat of mouse mammary tumor virus (MMTV LTR) of pMAM was designated as pMLGT.

Cell culture and expression of GLUT2

CHO cells were cultured in Ham F-12 medium containing 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 mg/ml) in 5% CO_2. When CHO cells reached near 70% confluence, pMLGT and pECE-IR were transfected into CHO cells by the calcium phosphate method as described previously (Gorman, 1985). After a 48 hr culture, the expression of the GLUT2 gene of pMLGT was induced by adding dexamethasone in the culture medium to a final concentration of 1mM. After a 17 hr incubation at 37℃, CHO cells were incubated in a fresh serum-free Ham F-12 medium for 2 hr at 37℃. After rapid washing with Krebs-Ringer-phosphate (KRP) buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.0 mM $CaCl_2$, 5.0 mM NaH_2PO_4, pH 7.4) equilibrated at 37℃, CHO cells were treated with or without 10^{-7} M insulin in KRP buffer for 30 min. The cells were washed three times with ice-cold phosphate-buffered saline and were scraped off from the flask bottom with a plastic cell scraper.

Subcellular fractionation of CHO cells

The plasma membrane fraction was obtained by the slight modification of the method described by Jarett (1974). Cells were homogenized using a tissue homogenizer (OMNI 5000) in the homogenization buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, and 1 mM phenylmethyl sulfonylfluoride) at speed 6 for 1.5 min. All steps were carried out at 4℃. The homogenate was centrifuged at 16,000xg for 15 min. The pellet was resuspended in the homogenization buffer and centrifuged at 1,000 xg for 10 min. The supernatant was centrifuged at 17,000 xg for 20 min to obtain the membrane fraction containing the plasma membrane. All samples were stored at −70℃. The quantity of protein was measured according to the method described by Lowry *et al.* (1951).

Western blot analysis

The expression of GLUT2 in CHO cells was identified by western blot analysis as described by Towbin *et al.* (1979). Membrane samples were mixed with the sample loading buffer and were incubated at 37℃ for 30 min. The mixture was subjected to SDS-polyacrylamide (8%) gel electrophoresis (Laemmli, 1970). Proteins in the gel were electrotransferred to a nitrocellulose (NC) membrane. The NC membrane was blocked with 10% nonfat dried milk in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20 (TBST) and was incubated for 1 hr with an antibody (1 : 500

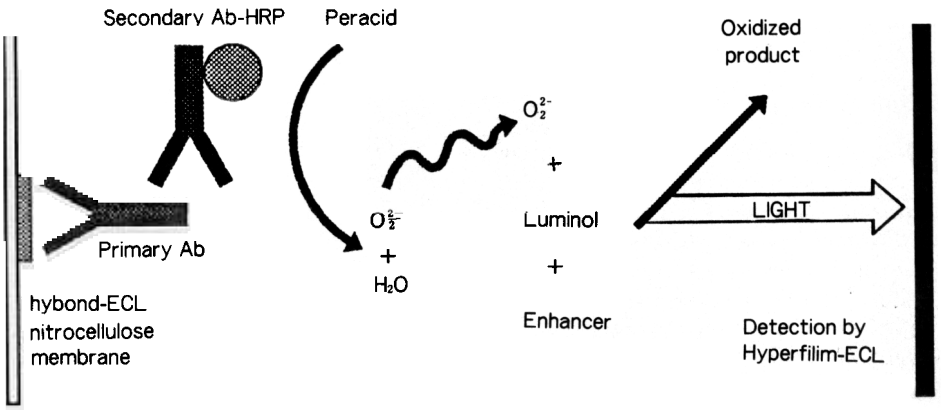

***Fig. 2.** The principle of ECL Western blotting.*

dilution) against C-terminal peptide (H_2N-Cys-Met-Glu-Phe-Leu-Gly-Ser-Ser-Glu-Thr-Val-COOH) of GLUT2 (Ahn *et al.* 1991). The quantity of GLUT2 protein expressed in the transfected CHO cells was expected to be so small that a newly developed protein detection method, Enhanced Chemiluminescence (ECL) method was used. The principle of the system is shown in Fig. 2. The NC membrane was incubated for 30 min with anti-rabbit IgG-horseradish peroxidase conjugate (1 : 5000 dilution) in TBST. To generate a signal, the NC membrane was incubated with ECL detection reagents for 1 min, and the signal was exposed to the Hyperfilm-ECL (Amersham). The intensity of the signal was measured by laser densitometer (2202 Ultroscan, LKB).

RESULTS

Amplification of GLUT2 cDNA by RT-PCR

Fig. 3 shows the GLUT2 cDNA products amplified by RT-PCR using primers 1 and 2 (5'-side fragment of GLUT2), and primers 3 and 4 (3'-side fragment of GLUT2). Lanes 2 and 3 represent the amplified products using cDNA synthesized from mRNA, and lanes 4 and 5 show the amplified products using cDNA synthesized from total RNA. In both cases, the DNA products represented the expected size (1,176 bp for 5'-side fragment and

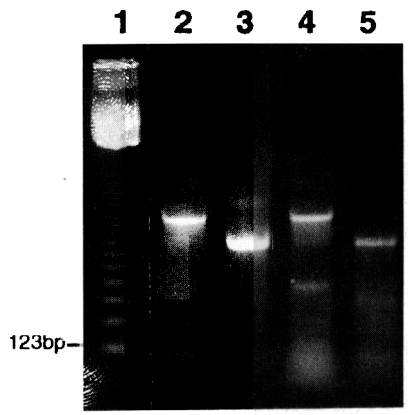

***Fig. 3.** Amplification of GLUT2 cDNA amplified by RT-PCR. The reaction mixture was subjected to agarose gel (1%) electrophoresis for 1 hr at 100V in TAE (Tris-Acetate-EDTA, pH8.0) buffer. GLUT2 cDNA fragments were amplified from mRNA (lane 2) or total RNA (lane 4) using primers 1 and 2, or from mRNA (lane 3) or total RNA (lane 5) using primers 3 and 4. Lane 1 indicates 123 bp DNA ladder. Primer 1, TGAGACAACAACTCCGCACACA (-24-1, sense); primer 2, TCCCAGCGACATGAAGACGGC (1152-1132, antisense); primer 3, GTCAGGGCAAAGAAAAGCTTG (754-774, sense); primer 4, GGAAGCTTAGGCAGCTCATCCTCACAC (1590-1564, antisense).*

837 bp for 3'-side fragment), although some minor DNA bands were visible in the pro-

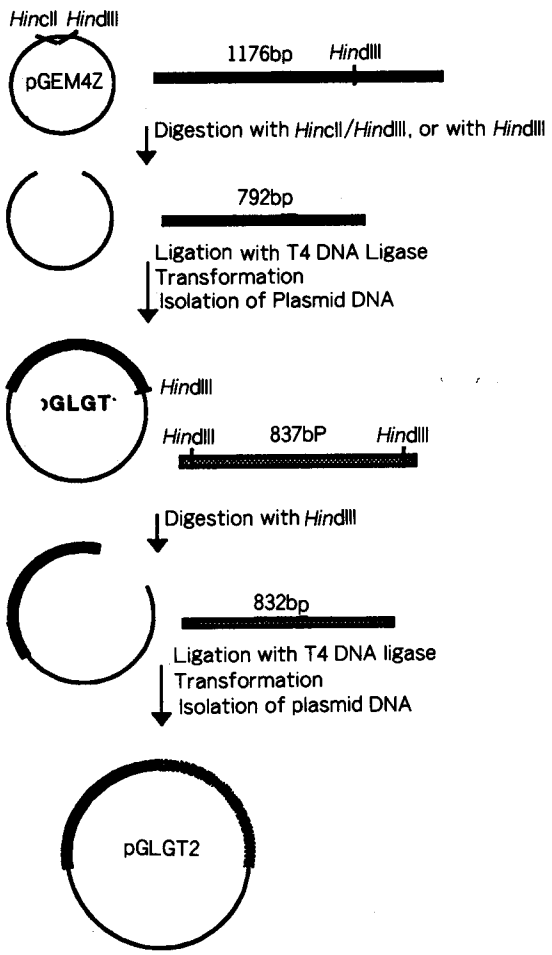

Fig. 4. *Cloning of PCR-amplified GLUT2 cDNA fragments.*

ducts of the total RNA template.

Cloning of GLUT2 cDNA

The strategy to construct pGLGT2 containing an entire open reading frame of GLUT2 cDNA is shown in Fig. 4. The correct linkage between 5'-fragment and 3'-fragment of GLUT2 cDNA in pGLGT2 was confirmed by restriction enzyme analysis (Fig. 5). The sizes of DNA fragments produced by restriction enzyme (*Hin*dIII, *Bam*HI, or *Hin*cII) digestion matched exactly with those which can be expected from the nucleotide sequence of GLUT2 cDNA.

Cloning of the GLUT2 cDNA in pMAM and the orientation of the GLUT2 cDNA that can be expressed within pMLGT was confirmed by digestion with *Sma*I and *Sal*I followed by agarose gel electrophoresis (Fig. 6).

Expression of GLUT2 cDNA in CHO cells

Since CHO cells are known to have small numbers of insulin receptor on their surface (Sung *et al.* 1992), pECE-IR containing the insulin receptor gene was cotransfected with pMLGT to get a maximal insulin effect. GLUT2 expressed in the plasma membrane fraction was detected by Western blot analysis (Fig. 7-A) and relative amounts of it in different groups were compared by densitometry scanning (Fig. 7-B). CHO cells that did not harbor pMLGT showed a minimal amount of intrinsic GLUT2 (lane 1). In contrast, CHO cells transfected with pMLGT (lane 2) showed a 2.9-fold increase in the expression of GLUT2 compared to the untransfected CHO cells, suggesting that the transient expression of GLUT2 cDNA was successful. The insulin treatment on the transfected CHO cells induced the 3.8-fold increase of GLUT2 in the plasma membrane compared to the non-insulin treated group (lane 3) and an 11-fold increase compared to untransfected CHO cells (lane 1). GLUT2 expressed in the plasma membranes of rat hepatocytes was shown in lane 4. These results suggest that GLUT2 expressed in CHO cells can be translocated by insulin.

DISCUSSION

Since the development of cDNA cloning techniques, many genes are cloned in the form of cDNA and expressed in eukaryotic or prokaryotic cells (Gubler and Hoffman, 1983). By this way, the structure and the function of specific proteins within cells have been worked out. Once the cDNA has been cloned and published, the cDNA should be sent to a researcher who wants to study further about the gene. However, in reality, this rule is not being followed for many reasons. In these cases, a researcher should clone the

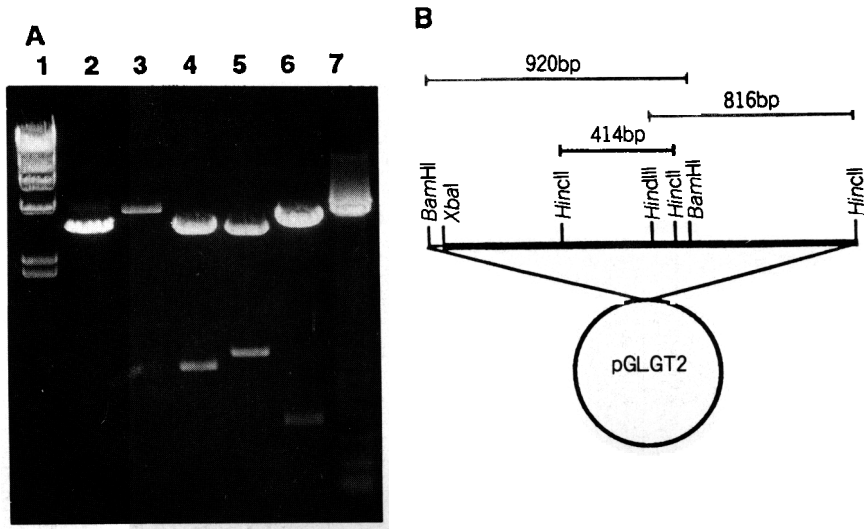

Fig. 5. *Restriction enzyme analysis of pGLGT2. pGLGT2 was digested with various restriction enzymes and subjected to agarose gel (1%) electrophoresis (panel A). Lane 1, λ DNA digested with HindIII; lane 2, pGLGT1 digested with HindIII; lane 3, pGLGT2 digested with SalI; lane 4, pGLGT2 digested with HindIII; lane 5, pGLGT2 digested with BamHI; lane 6, pGLGT2 digested with HincII; lane 7, 123bp DNA ladder. Panel B shows a schematic diagram of restriction map of GLUT2 cDNA.*

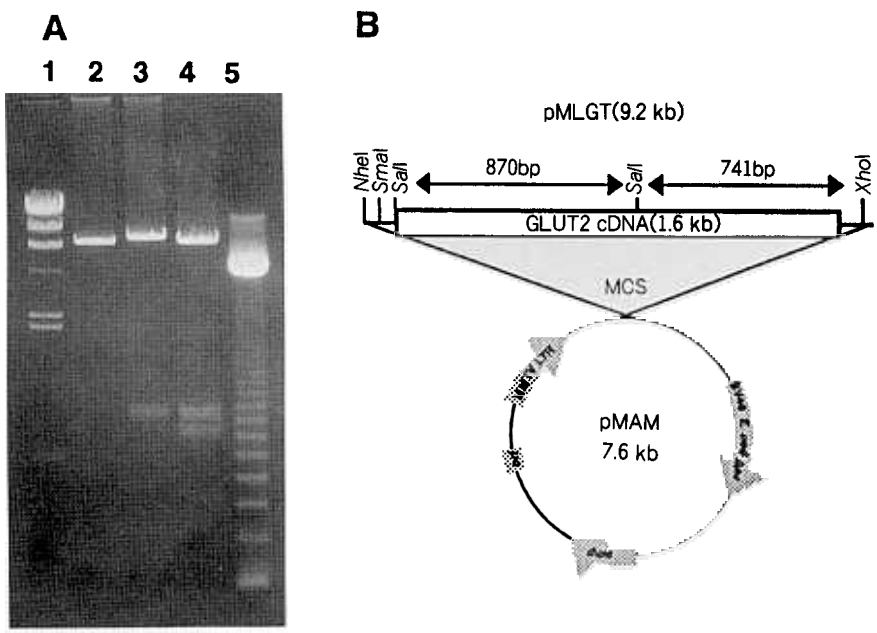

Fig. 6. *The confirmation of the orientation of GLUT2 cDNA in the recombinant plasmid DNA (pMLGT). pMAM containing GLUT2 cDNA was digested with SmaI/SalI and was subjected to agarose gel (1%) electrophoresis (panel A). Panel B shows a schematic diagram showing the structure of pMIGT. Lane 1, λ DNA digested with HindIII; lane 2, pMAM digested with SmaI/SalI; lane 3, recombinant plasmid (clone 1) digested with SmaI/SalI; lane 4, recombinant plasmid (clone 2) digested with SmaI/SalI; lane 5, 123bp DNA ladder. A recombinant plasmid from clone 1, which has a correct orientation of GLUT2 cDNA within pMAM, was designated as pMLGT.*

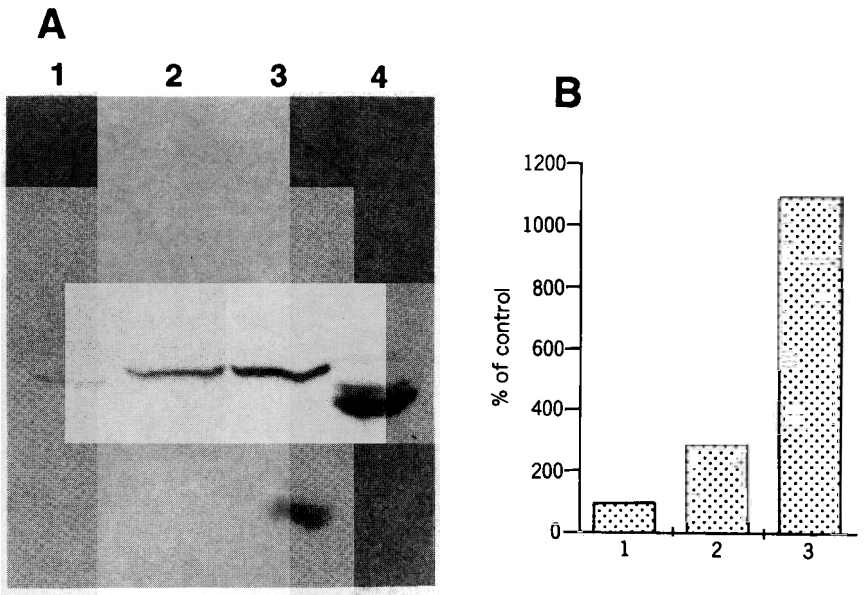

Fig. 7. Effect of insulin on the expression of GLUT2 in the plasma membrane fraction of CHO cells. CHO cells grown to 70% confluence were transfected with pMLGT and dexamethasone (1 mM) was added into the culture medium. The cells were washed with KRP buffer and incubated with or without insulin (10^{-7}M) for 30 min. The cells were washed with ice-cold PBS and were scraped off from the bottom of culture flask. The plasma membrane fraction was obtained by the method of Jarett (1974) and GLUT2 present in the plasma membrane was identified by Western blot analysis using peptide-specific antibody against GLUT2 (panel A). Panel B shows the result of the densitometric scanning of the signals shown in panel A. Lane 1, CHO cells; lane 2, pMLGT-transfected CHO cells, no insulin treated; lane 3, pMLGT-transfected CHO cells, insulin treated; lane 4, rat hepatocyte.

cDNA from the beginning by himself. Recently, the advancement of PCR technology (Saiki *et al.* 1985) made it easy to amplify the specific DNA fragments from the published nucleotide sequences.

In this study, GLUT2 cDNA was amplified by RT-PCR from the published sequence data (Fukumoto *et al.* 1988) and cloned into pMAM to construct pMLGT, of which GLUT2 cDNA could be expressed in CHO cells. Although the CHO cells have been known to be sensitive to insulin action, the number of insulin receptors in the plasma membrane is low(~5,000, Sung *et al.* 1992). We have transfected insulin receptor cDNA together with pMLGT into CHO cells to test the possible translocation of GLUT2 protein that is not translocated in the cells of its major distribution, such as pancreatic β cells or hepatocytes. Although the translocation of GLUT4 in muscles or adipocytes are well established, the other types of glucose transporters do not respond to insulin when they are present in tissues of their major distribution. Recently, it was reported that the GLUT1 protein is also present in rat adipocytes and also translocate along with GLUT4 when they are exposed to insulin (Oka *et al.* 1988) although the extent of responses was different. After transfection and expression of GLUT1 cDNA in CHO cells (Asano *et al.* 1989) and 3T3-L1 adipocytes (Gould *et al.* 1989), the successful recruitment of GLUT1 protein in response to insulin was observed. These results indicate that the translocation from the intracellular compartment to the plasma membrane fraction depends on some tissue-specific signaling mechanism which is still unknown to us rather than on an intrinsic property of the various

types of glucose transporters themselves. In order to generalize this hypothesis, an examination on the translocation of other types of glucose transporters that are not responsive to insulin is necessary.

In this study, insulin-insensitive GLUT2 protein was expressed in CHO cells together with insulin receptors to augment insulin response. The expression of GLUT2 protein in the plasma membrane fraction in transfected CHO cells increased 2.9-fold compared to that in untransfected CHO cells, indicating the successful transfection and expression within the cells. The translocation of GLUT2 was increased 3.8-fold by insulin treatment compared to that of the non-insulin treated group and 11-fold compared to that of CHO cells which was used as a negative control. From these results, it is assumed that the translocation of GLUT2 can occur if the protein is expressed in cells that respond to insulin action. When the GLUT1 was expressed in 3T3-L1 adipocyte, the magnitude of insulin mediated translocation as measured by peptide-specific antibody was 1.5~2.0 fold (Gould *et al.* 1989) and this observation is in good agreement with other reports (Baly and Horuk, 1987; Gibbs *et al.* 1988). Our result showed the 3.8-fold increase in GLUT2 in the insulin treated group compared to that of the non-insulin treated group. This difference in the magnitude of translocation may be originated from (1) differences in cell types used in experiment or; (2) the effect of transfected insulin receptor in order to get the maximal insulin effect, because CHO cells are known to have a relatively low number of insulin receptors.

Taken together, the results shown here and the data from GLUT1 translocation, support the hypothesis that the translocation of glucose transporters occurs by insulin if they are put into insulin sensitive cells, although the immediate downstream signal that mediates this translocation phenomena is still unknown.

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