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A Study on the Regulation of Translocation of Glucose Transporters during Hepatocarcinogenesis Induced by 3'-Me DAB

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The mechanism of glucose transporter(GT) expression on the plasma membranes of hepatoma cells in rats induced by 3'-methyl-4-dimethylaminoazobenzene (3'-Me DAB) was studied. Cytochalasin B binding to plasma membrane fractions from control and 3'-Me DAB group in the absence of cold cytochalasin B showed 9,825±925 and 30,165±625 dpm/mg membrane protein. Scatchard plot analysis showed that the GTs present on the plasma membrane fractions in control and 3'-Me DAB groups were 5.0 and 16.0 pmol/mg membrane protein and their Kd values were 151 and 157 nM, respectively. These results suggest that the numbers of GTs in plasma membrane were increased in the3'-Me DAB group compared to the control group. In contrast, the amounts of GTs in low density microsomal (LDM) fractions measured by a photoaffinity labeling technique using [^{3}H]-cytochalasin B were 31,207 and 11,702 dpm/mg protein in the control and 3'-Me DAB group, respectively. These results suggest that GTs were translocated from LDM to plasma membranes during carcinogenesis. To confirm these results by an independent method 10% SDS-polyacrylamide gel electrophoresis was carried out. Gel slice No. 13 corresponding to MW of 45 kDa from plasma membrane fractions showed increased radioactivities in the 3'-Me DAB group compared to the control group. However, LDM fractions of the 3'-Me DAB group showed decreased radioactivities compared to the control group. Western blot analysis using anti-human RBC GT antibody present in the plasma membranes and LDM fractions from control and 3'-Me DAB groups did not show any significant difference, indicating low cross-reactivity between them. These results indicate that increased glucose transport seems to be more likely due to reciprocal redistribution of GTs between plasma membrane and LDM fractions.

Key Words: Western blot, plasma membrane, low density microsomal fractions, glucose transporter, hepatocarcinogenesis, 3'-Me DAB, translocation

The concentration of blood glucose is regulated by the metabolic interplay of liver and peripheral tissues such as adipose tissues, skeletal muscles, and cardiac muscles. The uptake of glucose into most cells is accomplished by a stereospecific, carrier-mediated transport system capable of transporting glucose down its concentration gradient (Ferrannini *et al.*, 1985; Wheeler and Hinkle, 1985). Although glucose transport in the adipose or muscle tissue is known to be stimulated by insulin (Levine and Goldstein, 1955), the transport into the liver cell is an insulin-independent process (Williams *et al.*, 1968; Baur and Heldt, 1977). The glucose transporters (GTs) involved in the liver are also known to be different in their primary structures, physical and chemical characteristics from the other types of GTs present in red blood cells (RBC) or muscle cells, and a detailed study on the kinetic properties of the liver GTs still needs to be explored.

Many studies have indicated that transporter proteins present in the plasma membranes might be changed during carcinogenesis by chemicals or a tumor-inducing virus (Suss *et al.*, 1972; Levine and Hassid, 1977; Lee and Weinstein, 1979; Castagna *et al.*, 1982). The increased glucose transport into cells (Lee and Weinstein, 1979) is one of the earliest

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changes observed during carcinogenesis. The possible involvement of pp60src in the increased expression of GT has been suggested (Inui *et al.*, 1980). The increased number of GTs suggested here is consistent with the increased Vmax without changes in K_M (Lee and Weinstein, 1979) in transformed cells.

Cytochalasin B, which is known to be a potent competitive inhibitor that binds to GT in a 1:1 molar ratio, has been widely used to quantitate and identify GT (Salter and Weber, 1979; Suzuki and Kono, 1980; Kono *et al.*, 1981; Pessin *et al.*, 1982; Oka and Czech, 1984). Kitagawa *et al.* (1985a) reported the increased translocation of GT from microsomes to plasma membranes in the 12-0-tetradecanoylphorbol-13-acetate (TPA) treated Swiss 3T3 cells. Recently Kim *et al.* (1987) reported that the plasma membrane extracts from 3'-methyl-4-dimethyl-aminoazobenzene (3'-Me DAB) induced hepatoma cells showed increased glucose transport activity compared to those from control group. However, they could not show any noticeable differences in GT activities in microsomes from hepatoma cells. The present study was designed to investigate the possible relationship between increased glucose transport and the translocation of GTs from low density microsomal (LDM) fractions to plasma membranes by photoaffinity labeling technique.

MATERIALS AND METHODS

Animals and diets

One hundred and twenty Albino male rats (~80g) were grouped into control and 3'-Me DAB induced hepatoma groups. Both groups received a control diet for two weeks. The diet for the 3'-Me DAB group was subsequently supplemented with 0.06% 3'-Me DAB (Miller *et al.*, 1948) for 12 weeks and then they were maintained with a control diet for the next 4 weeks. The control group received the control diet during the entire experimental period (Table 1).

Table 1. Composition of the diets (per kg)

Constituent	Diet group	
	Control	3'-Me DAB
Casein, g	180	180
Corn oil, g	50	50
Glucose monohydrate, g	770	770
Salt mixture, g	40	40
Riboflavin, g	0.001	0.001
Vitamin mixture*, ml	5	5
3'-Me DAB, g	–	0.6

* Contains vitamin A 20,000 units, vitamin D 2,000 units, choline chloride 1.5g, pteroyl glutamic acid 0.6g, biotin 1.5mg, thiamine-HCl 20mg, pyridoxine-HCl 20mg, menadione 50mg, nicotinamide 50mg, potassium parabenzoic acid 50mg, calcium cyanocobalamine 40μg.

Fractionation of plasma membrane and LDM from rat liver

Plasma membranes were fractionated by the method of Axelrod and Pilch (1983). All the experimental procedures were carried out at 4°C. Control and 3'-Me DAB group rats were sacrificed under ether anesthesia and livers were perfused with ice-cold physiological saline to remove blood. Livers from both groups were weighed, minced and homogenized in 4 volumes of 0.25M sucrose in 10mM N-2-hydroxyethyl-1-piperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.4 with a Teflon homogenizer. The homogenates were spun for 10 min at 150xg to remove cell debris and nuclei. The supernatants were centrifuged for 10 min at 2,000xg. Supernatants were saved for the fractionation of LDM. For the isolation of plasma membrane, the precipitates were resuspended in 70% (W/W) sucrose solution to make 47% (W/W). Then 37% (W/W) sucrose and 0.25M sucrose in 10mM Hepes, pH 7.4 solutions were overlaid onto the 47% (W/W) sucrose suspension and the tube was centrifuged at 100,000xg for 3 hours. The plasma membranes located between the 37% sucrose and 0.25M sucrose layers were harvested, then centrifuged at 40,000xg for 30 min. The precipitates were resuspended in 10mM Hepes, pH 7.4 and once more homogenized with a glass homogenizer and saved for use as plasma membrane fractions. 5'-Nucleotidase activity was measured to check the purity of the plasma membrane fractions by the method of Heppel and Hilmoe (1955). Protein concentration was measured by the method of Lowry *et al.* (1951). LDM fractions were isolated by the method of Fleischer and Kervina (1974). The supernatant fluids saved for LDM fractions were centrifuged at 220,000xg for 30 min. The supernatants were centrifuged for 90 min at 220,000xg. The resulting pellets were washed with 50mM sodium phosphate buffer, pH 7.4 and resuspended with the same buffer in the concentration range of 1-5 mg protein/ml.

Cytochalasin B binding assay in rat liver cells

The quantitation of GTs present on the plasma

membrane was carried out by measuring the binding of cytochalasin B as described by Axelrod and Pilch (1983). Plasma membrane fractions from control and 3'-Me DAB groups were washed twice with Krebs-Ringer phosphate buffer (128mM NaCl, 5.2mM KCl, 1.4mM $CaCl_2$, 1.4mM $MgSO_4$, 10mM Na_2HPO_4, pH 7.4) and used for the cytochalasin B binding study . The 0.2mg protein of plasma membrane fractions was incubated in the presence of 5μM cytochalasin E and 12.7nM [^{3}H]-cytochalasin B and cold cytochalasin B was added to give final concentrations of up to 1,600nM. Final ethanol concentration in the assay mixture was adjusted to 1%. The reaction was stopped by centrifuging at 12,000 rpm for 5 min. The pellets were washed with cold Krebs-Ringer phosphate solution containing 1% ethanol to remove unbound [^{3}H]-cytochalasin B. Pellets were removed by cutting the tip of the microcentrifuge tube and radioactivities of both pellets and supernatant fluid were measured in a liquid scintillation counter. Amounts of GTs present in LDM fractions were measured by the method of photoaffinity labeling (Ciaraldi *et al.*, 1986). The 0.5mg proteins of LDM fractions from both groups were incubated in the presence of 126nM [^{3}H]-cytochalasin B for 10 min at 4°C in reaction quartz cuvettes. 400μM hydroxysuccinimidyl-4-azidobenzoate (HSAB) was added to the reaction mixture and irradiated with a 100 W UV lamp (254nm) for 5 min at a distance of 10cm above the reaction cuvette. The reaction was stopped by adding 1ml of 1 N NaOH and precipitated by adding 3ml of 10% TCA. The pellets were obtained by centrifugation at 23,000xg for 10 min and washed again with 10% TCA solution. The pellets were dissolved with 200 μl of 1 N NaOH and radioactivities were measured in a liquid scintillation counter.

Molecullar weight determination of glucose transporters present in the plasma membranes and LDM fractions

Plasma membrane and LDM fractions were incubated with [^{3}H]-cytochalasin B and cross-linked with UV -irradiation using HSAB by the method described above. These proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970). Gels were sliced into 2 mm in length and incubated in 400μl H_2O_2 at 50-60°C for 16 hours, and the radioactivities were measured in a liquid scintillation counter.

Western blot analysis of plasma membranes and LDM fractions from control and 3'-Me DAB groups

The plasma membranes and LDM fraction samples from both groups were subjected to 5-17% linear gradient SDS-PAGE and transferred to nitrocellulose (NC) membranes (Towbin *et al.*, 1979). NC membranes were incubated with 1:500 diluted antibodies against human RBC-GT prepared by Lee *et al.* (1990) and the colors were developed with goat anti-rabbit IgG-alkaline phosphatase conjugate (Promega Co., WI, USA).

RESULTS

Quantitation of glucose transporters in the plasma membranes of rat liver

[^{3}H]-Cytochalasin B binding to liver plasma membrane from control and 3'-Me DAB groups is shown in Fig. 1. Cytochalasin B binding in the absence of cold cytochalasin B was 9,825±925 and 30,165±625 dpm/mg membrane protein, respectively. There is a 3.2-fold increase in binding of cytochalasin B in the 3'-Me DAB group compared to the control group. Cold cytochalasin B markedly inhibited [^{3}H]-cytochalasin B binding to plasma membranes from both groups, which indicates that they are competing with each other. Fig. 2 shows the Scatchard analysis of Fig. 1 data. The calculated total number of binding sites and Kd values for control and 3'-Me DAB groups were summarized in Table 2. The number of binding sites in the 3'-Me DAB group was increased 3.2 times compared to that of the control group. However, no changes were observed in Kd values between the two groups.

These results indicate that increased glucose transport into hepatoma cells is not due to their increased affinity for glucose but due to the increased GTs present on the plasma membranes.

Quantitation of glucose transporters in the LDM fraction of rat liver

Cytochalasin B binding to LDM fractions in the 3'-Me DAB group was one-third of that of the control group (Table 3). These data show the exact contrast of those observed in the plasma membranes of control group being 3-fold of that in 3'-MeDAB group. These results (Tables 2, 3) suggest that the increased GTs in plasma membrane and decreased GTs in the LDM of 3'-Me DAB group were due to the increased translocation of GTs from LDM to plasma membrane during hepatocarcinogenesis.

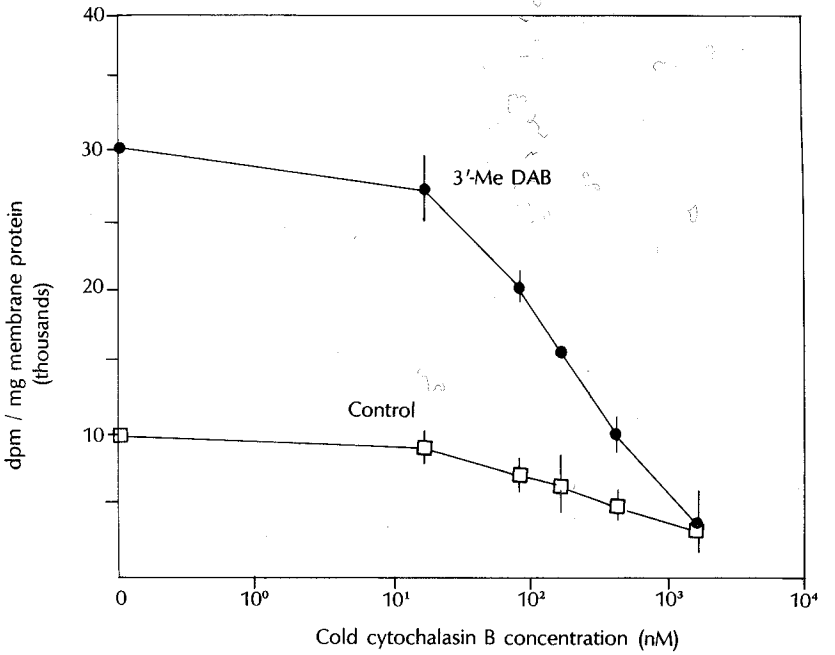

Fig. 1. *Cytochalasin B binding to the plasma membrane from control and 3'-Me DAB group. Plasma membranes were incubated for I hour at 22°C in the presence of 5μM cytochalasin E, 12.7nM [³H]-cytochalasin B, and cold cytochalasin B was added to final concentrations ranging from 0 to 1,600nM.*

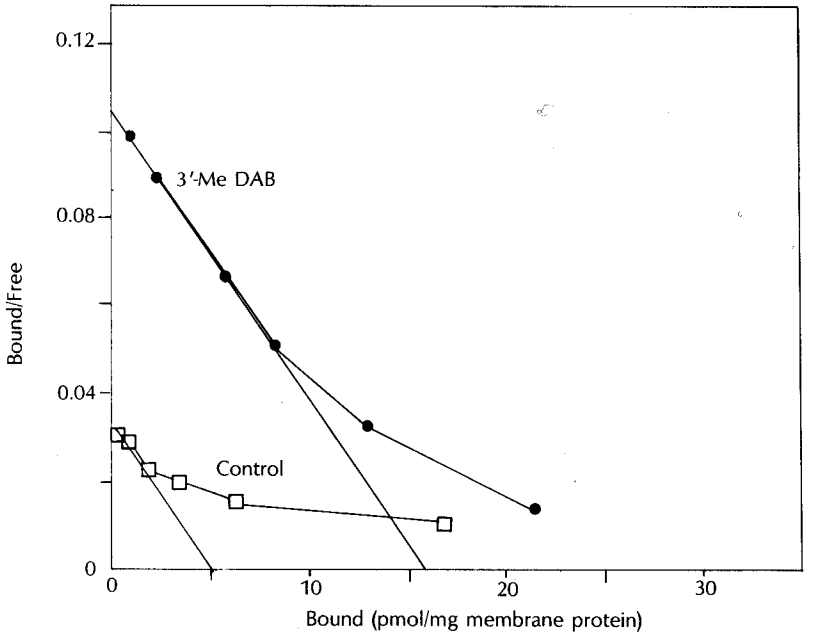

Fig. 2. *Scatchard analysis of D-glucose inhibitable cytochalasin B binding to the plasma membrane prepared form rat liver.*
□ *Control*
● *3'-Me DAB*

Table 2. Distribution of cytochalasin B binding sites to rat liver plasma membranes

Group	Ro	Kd
Control	5.0±1.4	151±17
3'-Me DAB	16.0±4.2	157±20

Ro: binding-site number (pmol/mg membrane protein)
Kd: dissociation constant (nM)
Results were obtained by Scatchard analysis.
Values are Mean±S.D. (n=3).

Table 3. Photoaffinity labeling of glucose transporters on the low density microsome from control and 3'-Me DAB groups. 0.5mg of low density microsomes from each group was mixed with 126nM [^{3}H]-cytochalasin B and cross-linked by UV (254nm) irradiation. Detailed methods were described in the text

	Control	3'-Me DAB
Irradiation	42,242±3,825*	27,776±5,577
Non-irradiation	11,035±1,375	16,074±1,133
Differences:	31,207	11,702

* Values represent Mean±S.D. (DPM/0.5mg LDM protein, n=3).

Molecular weight determination of glucose transporters present in the plasma membranes and LDM fractions

Photoaffinity labeling of the plasma membrane showed a major radioactivity peak, migrating with a molecular weight of 45 kDa. The radioactivity associated with this peak from the 3'-Me DAB group was higher than that from the control group (Fig. 3). These data correlate well with the kinetic data of the Scatchard plot in that the plasma membrane of the 3'-Me DAB group showed an increased binding of

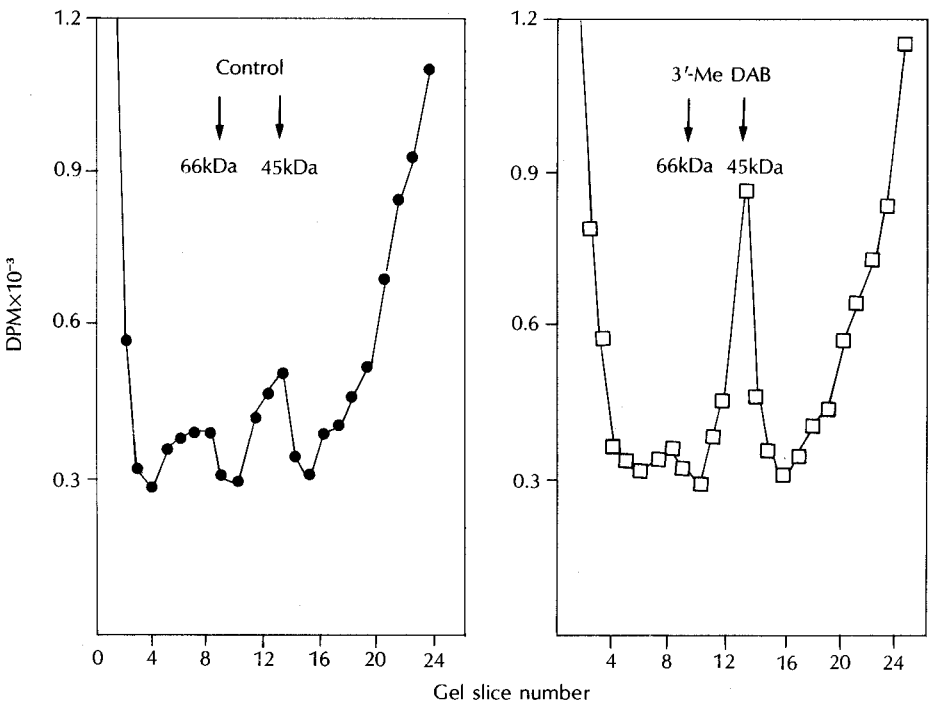

Fig. 3. *Electrophoretic profile of [^{3}H]-cytochalasin B labeled liver plasma membranes by photoaffinity labeling. Plasma membranes of control and 3'-Me DAB were incubated with [^{3}H] cytochalasin B for 10 min at 4°C. Hydroxysuccinimidyl-4-azidobenzoate was then added and after incubation in the dark for 5 min the membranes were irradiated with UV light (254nm) for 2 min. Membrane protein was applied to 10% gels and analyzed by SDS-PAGE. Arrows indicate the position of marker protein.*

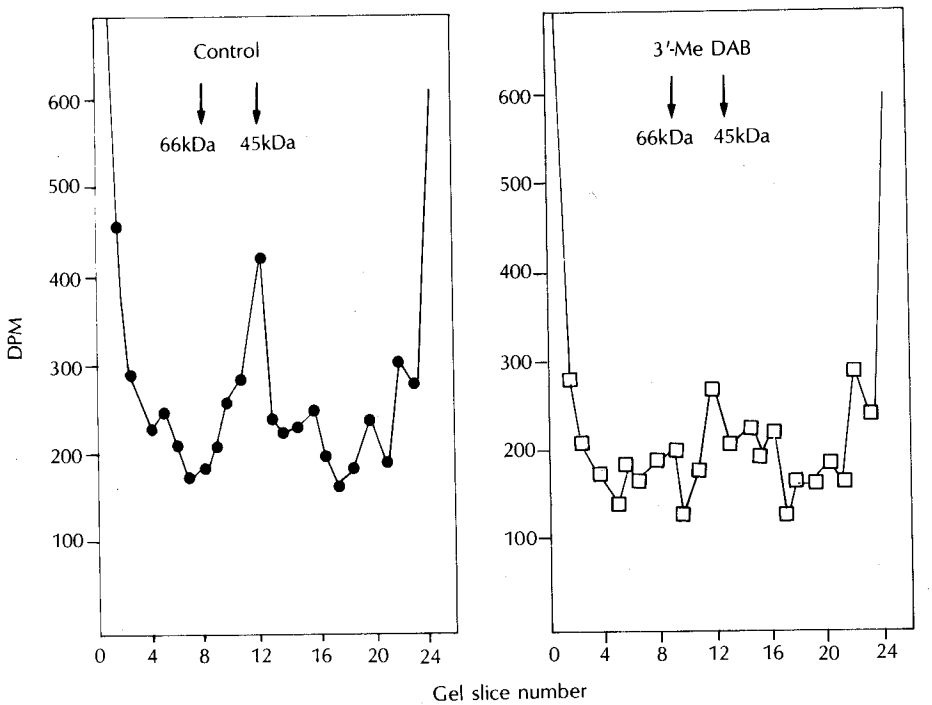

Fig. 4. *Electrophoretic profile of [³H]-cytochalasin B labeled low density microsome by photoaffinity labeling.*

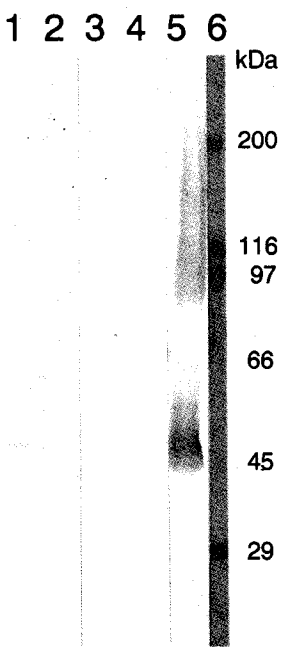

Fig. 5. *Immunoblot analysis of plasma membranes and LDM from rat liver by polyclonal antibody against human RBC-GT.*

Plasma membranes (Lanes 1, 2) and LDM (Lanes 3, 4) from control and 3'-Me DAB group liver were subjected to 5-17% SDS-PAGE and the proteins were electroblotted onto nitrocellulose membrane. The nitrocellulose membrane was treated with polyclonal antibody against human RBC-GT and colors were developed using goat anti-rabbit IgG alkaline phosphatase conjugate.

Lanes 1, 2: Plasma membranes (60μg) from control and 3'-Me DAB group

Lanes 3, 4: LDM (60μg) from control and 3'-Me DAB group

Lane 5: Protein depleted membrane (30μg) from human RBC

Lane 6: Molecular weight standards

cytochalasin B without changing its affinity. In contrast, a similar 45 kDa protein band in the 3'-Me DAB group was markedly reduced compared to that of LDM in the control group (Fig. 4). These results clearly indicate the translocation of GT from LDM fractions to plasma membranes during hepatocarcinogenesis. Also, it was observed that there is a heterogeneity in GTs in LDM. This is in contrast to the plasma membrane transporters which are more uniform in charge properties. The difference between GTs in plasma membrane and LDM fractions may be attributed to the differences in glycosylation of GTs present in these subcellular fractions.

Western blot analysis of plasma membranes and LDM fractions

Plasma membrane and LDM proteins were prepared from both groups and subjected to 5-17% SDS-PAGE. After transferring the proteins to NC membrane, they were immunostained using polyclonal antibody against human RBC-GT.

Immunoblot analysis of protein depleted membranes prepared from human RBC using polyclonal antibody indicated that the antibody was prepared properly (Fig. 5, Lane 5). Although the signals were not strong, GTs were clearly detected on the liver plasma membrane fractions of the control and 3'-Me DAB groups (Fig. 5, Lanes 1, 2), indicating that low cross-reactivity between human RBC-GT and rat liver-GT is due to the difference in their primary structure. However, in the LDM fractions from both groups, no detectable band corresponding to GT was observed (Fig. 5, Lane 3, 4).

DISCUSSION

For the unregulated cells, glucose is transported into cells rapidly to meet their metabolic needs (Weber, 1973; Salter and Weber, 1979). Although the liver is known to be the main organ of the regulation of glucose homeostasis in vivo, it is exceptional in that the glucose transport into liver cells is independent of insulin (Baur and Heldt, 1977; Craik and Elliott, 1979). When chicken embryonic cells were infected with Rous sarcoma virus (RSV), the activity (Inui *et al.*, 1985) as well as the amount (Salter *et al.*, 1982) of GTs were reported to be increased. A specific protein expressed during carcinogenesis might be involved in the transcription of GTs or activation of GTs in transformed cells.

In this experiment an attempt has been made to understand the mechanism of GT expression in the plasma membranes of hepatoma cells induced by 3'-Me DAB and antibody against purified human RBC-GT was used for western blot analysis to test the GTs expressed on the plasma and microsomal membranes of control and 3'-Me DAB groups. These results suggest that the GTs expressed in the LDM fraction of normal liver or hepatoma cells do not cross-react with human anti-RBC-GT antibody, or the GTs present on the LDM fraction of the rat liver were too scarce to be detected by the immunochemical method (Ciaraldi *et al.*, 1986). Therefore, no definite conclusion on the translocation of GT can be made. It can, however, be concluded that the GTs of normal or hepatoma cells does not cross-react with antibodies against GT from human RBC.

Mueckler *et al.* (1985) cloned a GT from HepG2 cells and suggested that RBC-GT was expressed in the hepatoma cell line. However, monoclonal antibody against human RBC-GT did not show cross-reactivity with GT present in normal liver and some cases of human primary hepatoma (Rhoads *et al.*, 1988). Also, they observed that this isoform was expressed in cultured primary hepatocytes. In this experiment, we had a clear 55kDa protein band reacted with anti-human RBC-GT antibody in plasma membranes from both control and 3'-Me DAB groups, although the signal was not strong. It is possible that the band was a result of nonspecific reaction, but another possible explanation for this discrepancy is that the specificity of antibody from this study and that of Rhoads' group (1988) might differ. We could not detect cross-reacting GT from LDM fractions of control and 3'-Me DAB groups. This is probably because the GT present in the LDM fraction of rat liver is immunologically different from RBC-GT or too small (Ciaraldi *et al.*, 1986) to be detected by immunoblotting.

Scatchard analysis revealed that cytochalasin B binding to the plasma membrane GT from the 3'-Me DAB group was increased 3.2-fold compared to that of the control group. No significant difference in Kd values between control and 3'-Me DAB groups was observed. These results suggest that increased glucose transport during hepatocarcinogenesis is due to the increased number of GTs present in the plasma membranes of hepatoma cells. In contrast, cytochalasin B binding to LDM fraction in the 3'-Me DAB group was one-third of that of the control group.

From these results, it can be suggested that the increased GT in plasma membranes with concomitant reduction of GT in LDM fractions of the 3'-Me DAB group compared to the control group results from the translocation of GT from the LDM fraction to the

plasma membranes during carcinogenesis.

Similar redistribution of GTs was observed in insulin responsive normal cells (Cushman and Wardzala, 1980; Cushman *et al.*, 1984), TPA treated Swiss 3T3 cells (Kitagawa *et al.*, 1985a; 1985b), and virus induced transformation process (Birnbaum *et al.*, 1986). Ciaraldi *et al* (1986) suggested that only one type of GT having pI 6.05 was seen in both plasma membrane and LDM fractions from liver. In contrast, adipocyte plasma membrane shows a single peak GT, at pI 5.6, whereas the LDMs display two transporter isoforms, at pI 5.6 and 6.4. Horuk *et al.* (1986) suggested that insulin mediates the translocation of the pI 5.6 isoform to the cell surface, whereas the pI 6.4 isoform is unaffected by the hormone. The lack of the translocated pI 6.4 isoform in liver could also explain the non-responsiveness of liver glucose transporter. One possible reason for the difference in charge properties of GT between liver and adipocytes could be derived from differences in post-translational modifications such as glycosylation or phosphorylation (Horuk *et al.*, 1986). Another possibility is the existence of a GT multigene family in one cell type (Kayano *et al.*, 1988). From the study here, a reciprocal redistribution of GTs from LDM to plasma membranes fractions during hepatocarcinogenesis was observed. However, it is not certain whether those GTs expressed in normal and transformed hepatocytes are the same or different types. From the cloned genes from HepG2 and normal liver cells, the primary structures were deduced and proved to be different with each other (Fukumoto *et al.*, 1988); therefore they are expected to be different in physico-chemical properties. In the established human hepatoma cell line (HepG2), the type of GT found in RBC was overexpressed rather than those normally present in the human liver (Mueckler *et al.*, 1985). This finding provides us with a new way of thinking about GT expression during the transformation process in addition to GT translocation. To date, the liver type GT was not purified to homogeneity to provide some kinetic differences between RBC-GT and liver-GT. However, it is conceivable that the RBC-type has a higher affinity for glucose compared to liver type to the meet active metabolic needs of tumor cells. However. Kd values of cytochalasin B binding to GTs present in the plasma membrane fractions in control and 3′-Me DAB groups were 151 and 157nM, respectively, indicating that there is no difference between the two values ($p>0.05$). Analysis of cytochalasin B binding by affinity labeling and SDS-PAGE showed a major protein peak with a molecular weight of 45 kDa and radioactivities from the 3′-Me DAB group were higher than those of the control group in the plasma membrane fraction. In contrast, a similar 45 kDa protein band was observed in the LDM fraction and radioactivities from the 3′-Me DAB group were decreased compared to the control group. These data correlated well with those obtained by Scatchard analysis of the cytochalasin B binding study.

In general, the molecular weight of human RBC-GT is known to be 55 kDa (Kasahara and Hinkle, 1977), the molecular weight of insulin responsive muscle type GT deduced from the cloned gene is about 46 kDa (Birnbaum, 1989), and that from HepG2 cells is estimated to be 50 kDa (Thorens *et al.*, 1988). However, these proteins showed a difference in the degree of glycosylation and sometimes molecular weights are underestimated (Rizzolo *et al.*, 1976; Bonitz *et al.*, 1980) or overestimated (Leach *et al.*, 1980) in SDS-PAGE. In this experiment, a broad band around the molecular weight of 45 kDa was observed. Therefore, at the present time, it is not clear whether the GT has a molecular weight of 55 kDa. The difference in the degree of glycosylation of GTs present in plasma membranes and LDM fractions might explain the sharp and broad radioactivity peak in the SDS-PAGE. More detailed study is under way to confirm this phenomenon, and to identify which type of GT was expressed on the plasma membrane of 3′-Me DAB induced hepatoma.

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