Proc. Natl. Acad. Sci. USA
Vol. 77, No. 5, pp. 2542–2545, May 1980
Biochemistry

Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site

(hormone/fat cells/liposomes/Golgi apparatus)

KAZUO SUZUKI AND TETSURO KONO*

The Department of Physiology, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232

Communicated by Sidney P. Colowick, February 11, 1980

ABSTRACT **The glucose transport activity of fat cells was assayed in a cell-free system. The activity was solubilized and incorporated into egg-lecithin liposomes. The carrier-mediated glucose transport activity was estimated by subtracting the cytochalasin B-insensitive component from the total glucose uptake activity of the modified liposomes. When a crude microsomal preparation from fat cells was fractionated by sucrose density gradient centrifugation, two transport activities (peaks A and B) were separated. Peak A coincided with the peak of 5′-nucleotidase, a marker of the plasma membrane. Peak B appeared to coincide with the peak of UDPGal:*N*-acetylglucosamine galactosyltransferase, a marker of the Golgi apparatus. Peak A was considerably smaller than peak B under basal conditions. When cells were exposed to 1 nM insulin for 5 min before homogenization, the height of peak A increased whereas that of peak B decreased. Insulin had no significant effect on the galactosyltransferase activity. The K_m values of glucose transport facilitated by the activities in peaks A and B were both approximately 10–15 mM. These results imply that insulin facilitates translocation of the transport activity from an intracellular storage site to the plasma membrane.**

We previously reported that fat cells rapidly internalize cell-bound insulin (1). The internalized hormone is associated with a subcellular structure that is separable from the plasma membrane by sucrose density gradient centrifugation. Because both the insulin receptor and the hormone-sensitive glucose transport mechanism are thought to be localized in the plasma membrane, we felt it of interest to explore the possibility that cells might internalize the transport mechanism along with the insulin-receptor complex. As a result of this study, we obtained preliminary data indicating that the glucose transport activities of fat cells are associated with two different subcellular structures. Our present study was initiated to carry out further investigation on the nature of these two glucose transport activities.

MATERIALS AND METHODS

Cytochalasin B was purchased from Aldrich, blue dextran from Pharmacia, and crude *l*-α-phosphatidyl choline of egg yolk (type IX-E, approximately 60% pure, lot no. 88C-7070, referred to as egg lecithin in this report) from Sigma. The sources of other materials are described elsewhere (1).

Isolated fat cells were prepared by the collagenase method (2) from epididymal and perirenal adipose tissues of Sprague–Dawley rats (180–280 g). Freshly prepared cells were incubated with gentle shaking (30 cycles per min) for 30 min at 37°C in Krebs–Henseleit Hepes buffer (3) containing fraction V bovine serum albumin at 20 mg/ml and 2 mM glucose. The purpose of this incubation was to stabilize the basal transport activity (4). The incubation was continued 5 more min either in the presence or in the absence of 1 nM insulin. The cells were then homogenized and the crude microsomal fraction was separated by differential centrifugation as described (1), except that the second centrifugation was carried out for 60 min instead of 30 min. The crude microsomal preparation was further fractionated by sucrose density gradient centrifugation (15–45%, wt/wt), as described (1). The microsomal subfractions thus obtained (each 0.7 ml, contained approximately 0.5 mg of protein from 4–5 rats) were subjected (*i*) to the assays of protein and various marker enzymes and (*ii*) to the solubilization and subsequent reconstitution of the transport system. The reconstitution of the transport system was carried out by modification of the procedures developed by others (5–10), as described below.

For concentration and solubilization of the transport activity, portions of the aforementioned fractions (0.5 ml each) were mixed with two volumes of cold 10 mM Tris·HCl, pH 7.5 (buffer A). The heavy particles in fractions 1 through 12 were sedimented by centrifugation at 50,000 × *g*, and the light ones in fractions 13 through 16 at 220,000 × *g*, both for 2 hr. Each pellet was suspended in 0.21 ml of 2% sodium cholate in buffer A containing blue dextran at 0.5 mg/ml. The mixture was kept at 0°C for 30 min while being agitated with a Vortex mixer for a few seconds at 10-min intervals. The preparation was then centrifuged for 30 min at 48,000 × *g*.† The "solubilization" of protein in each fraction was approximately 60–70%.

For reconstitution of the transport system, 0.2 ml of the supernatant obtained by the above centrifugation was applied to a column of Sephadex G-50 (0.7 × 3 cm) equilibrated with buffer A. The column was washed with buffer A at room temperature, and 5 drops (280 mg = 280 μl) of the blue dextran fraction at the void volume were collected into a plastic test tube with an airtight stopper. The fraction was mixed with ¼ vol (i.e., 70 μl) of 5% egg lecithin that had previously been dispersed into buffer A by sonication and kept under an atmosphere of nitrogen. The test tube was filled with nitrogen and stoppered. The mixture was sonicated for 10 sec at 22–24°C in the cup horn of a Branson sonifier with an output of 75 W. The preparation was frozen once at −70°C, thawed, and sonicated again for 5 sec at 22–24°C with an output of 60 W. The preparation thus obtained is referred to as either modified liposomes or the reconstituted transport system.

For determination of the transport activity, samples of the modified liposomes (stable for at least 1 hr at 37°C) were warmed up to 37°C. Subsequently, a sample (containing 400

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* To whom correspondence should be addressed.

† When the preparation was centrifuged for 2 hr at 220,000 × *g*, a significant fraction of the transport activity was lost. On the other hand, the reconstitution was either greatly hampered or did not occur at all unless the preparation was "solubilized" with detergent and the "insoluble" substance was removed by centrifugation at 31,000 × *g* to 48,000 × *g* for 30 min. These results were obtained with crude microsomal preparations.

μg of egg lecithin) was incubated for 10 sec at 37°C in a total volume of 50 μl with 1 mM D-[^{3}H]glucose (10 μCi/ml; 1 Ci = 3.7 × 10^{10} becquerels)/10 mM Tris·HCl, pH 7.5/1% ethanol (the solvent of cytochalasin B) either in the presence or in the absence of 20 μM cytochalasin B. The reaction was terminated by the addition of 1 ml of ice-cold 1 mM $HgCl_2$ in buffer A (buffer B). The mixture was immediately filtered by suction with a Millipore filter (GSWP, 0.22 μm in pore size, 25 mm in diameter). The filter was then washed four times with 1-ml portions of buffer B. The time required for the filtration and washing was usually 20–30 sec. The radioactivity on the filter was measured by the liquid scintillation method.

As shown by others, modified liposomes would take up D-glucose by both carrier-mediated transport and non-specific "leakage" (5–10). However, the former is specifically blocked by cytochalasin B or $HgCl_2$ (5, 6, 9, 10). Therefore, we estimated the mediated transport activity by subtracting the cytochalasin B-insensitive component from the total transport activity. Unless otherwise noted, only this mediated transport activity is presented in this communication, and it is simply referred to as the transport activity.

UDPGal:*N*-acetylglucosamine galactosyltransferase was assayed by modification of the procedure described by Fleischer (11), and 5′-nucleotidase by that of Avruch and Wallach (12). Protein was determined by the method of Lowry *et al.* (13); the standard used was crystalline bovine serum albumin.

RESULTS

Fig. 1 shows the distribution of the glucose transport activities in the microsomal subfractions separated by sucrose density gradient centrifugation. The microsomal preparations were obtained from the control (basal) and insulin-treated fat cells. The data obtained with fractions 1 through 6 are not presented because no significant glucose transport activities were found in these fractions. As shown in Fig. 1 *a* and *b*, two glucose transport activities, designated as peaks A and B, were separated.‡ Peak A apparently coincided with a peak of protein (Fig. 1 *a* and *b*), which was associated with 5′-nucleotidase (Fig. 1 *c* and *d*), a marker enzyme of the plasma membrane (14, 15). Peak B seemingly coincided with the peak of UDPGal:*N*-acetylglucosamine galactosyltransferase (Fig. 1 *c* and *d*), a marker enzyme of the Golgi apparatus (11, 16). The sites of peaks A and B were distinct from the previously identified peak of mitochondria, which usually appears in fraction 5 (1, 17), the peaks of lysosomes, in fractions 5 and 15–16 (1, 18), the peak of the endoplasmic reticulum, in fractions 12–13 (1, 17), and that of the internalized insulin, in fractions 13–14 (1, 18).

The data in Fig. 1 further suggest that insulin had opposite effects on the heights of peaks A and B. The credibility of this observation was statistically tested in the subsequent experiments, and the results are summarized in Table 1. These results indicate that insulin significantly increased the height of peak A and decreased that of peak B ($P < 0.01$ in both cases). Although insulin lowered the height of peak B, it had no significant effect on that of the galactosyltransferase activity ($P > 0.1$). The data in this table further suggest that the effect of insulin on peak A was smaller than its effect on peak B. However, the validity of this comparison is debatable because these transport activities were those exhibited by the reconstituted systems, and it could be argued that the transport activity in peak A was more difficult to reconstitute than the activity in peak B. It should be pointed out, however, that the transport activity of the individual fraction was roughly proportional to the amount of the protein solubilized from either peak A or peak B and used for the reconstitution of the transport system (Fig. 2).

‡ The total recovery of the transport activities at the step of sucrose density gradient centrifugation was approximately 70–80%.

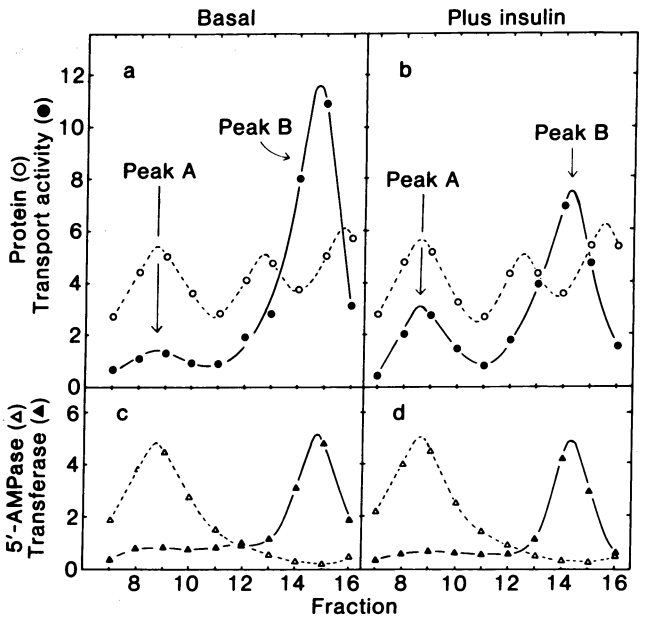

FIG. 1. Distribution of the glucose transport activities in microsomal subfractions of fat cells. Crude microsomal preparations were obtained from the basal (*a* and *c*) and insulin-treated fat cells (*b* and *d*) and further fractionated by sucrose density gradient centrifugation. The hormone treatment was done at 37°C for 5 min with 1 nM insulin. The sucrose fractions were analyzed for: glucose transport activity (●), protein (○), 5′-nucleotidase (5′-AMPase, △), and UDPGal:*N*-acetylglucosamine galactosyltransferase (transferase, ▲). The units of the activities shown are: nmol min^{-1} ml^{-1} × 10^{-1} for glucose transport, mg ml^{-1} × 5 for protein, nmol min^{-1} ml^{-1} × 0.15 for 5′-AMPase, and nmol min^{-1} ml^{-1} × 5 for transferase. The sucrose concentration in fraction 1 was 45% wt/wt, and that in fraction 16 was 15%. The indicated glucose transport activity was the cytochalasin B-sensitive portion of the transport activity exhibited by liposomes into which were incorporated materials solubilized from each sucrose fraction. The data presented in *a* and *c* were obtained by analyzing portions of the same samples, as were the data in *b* and *d*. Each point represents the mean value of two or three assays. The entire experiment (except the transferase assay) was repeated eight times with similar results.

Fig. 3 represents the time courses of glucose uptake by liposomes incorporating materials solubilized from either peak

Table 1. Effects of insulin on glucose transport activities and UDPGal:*N*-acetylglucosamine galactosyltransferase

	Glucose transport, nmol min^{-1} mg^{-1} of peak A protein		Transferase (peak B)
	Peak A	Peak B	
Basal	13 ± 2 (8)	115 ± 11 (8)	1.07 ± 0.03 (3)
Plus insulin	26 ± 3 (8)	69 ± 4 (8)	1.38 ± 0.20 (3)
Insulin effect	$P < 0.01$	$P < 0.01$	$P > 0.10$

Data are given as mean value ± SEM (*n*). Sixteen separate experiments (eight with and eight without insulin) were carried out as described for Fig. 1. The data were normalized by dividing the peak value of each activity by the peak concentration of protein in peak A, and the results were analyzed by Student's *t* test. We used the protein in peak A as the internal reference standard for normalization because this protein, which probably represents the plasma membrane protein (see *Discussion*), is separated from other protein peaks and is easily identifiable. In contrast, no protein peak that corresponds to peak B has been isolated.

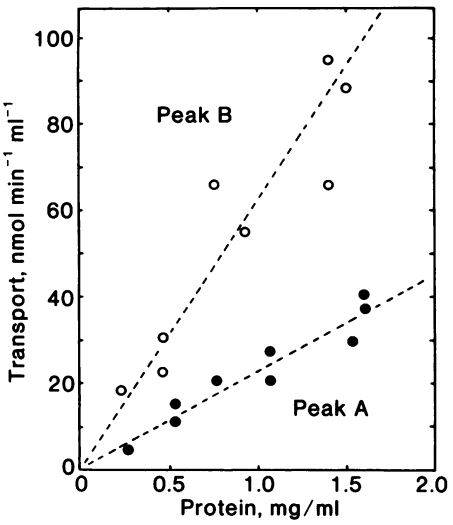

FIG. 2. Relationship between the protein concentration used for the reconstitution of liposomes and the glucose transport activity. The transport system was reconstituted by using the indicated concentrations of protein solubilized from either peak A or peak B of insulin-treated cells, and the glucose transport activity of the reconstituted system was determined. Values on both ordinate and abscissa of this figure are expressed per ml of the reaction mixture that was used for reconstitution of the transport system.

A or peak B. In either case, the total glucose uptake was considerably, but not entirely, inhibited by 20 μM cytochalasin B. Because this compound is a well-documented inhibitor of the carrier-mediated glucose transport (5, 6, 19, 20), these data imply that both peak A and peak B contained two types of activities: one that facilitates the carrier-mediated transport and the other that causes nonspecific "leakage." Incidentally, liposomes consisting of only egg lecithin did not take up glucose at any appreciable rate (data not shown).

The factors solubilized from either peak A or peak B also facilitated the release of D-glucose from liposomes that had been loaded with the sugar by sonication; however, the precise time courses of release were difficult to determine because, even after a long incubation, only about 20% of the total glucose in the liposomes was released (data not shown). It may be added in this regard that the maximum uptake of glucose was also only about 20% of the maximum incorporation achieved by the sonication method (data not shown). The meaning of this observation is not clear.

The apparent K_m values of glucose transport stimulated by the activities solubilized from peaks A and B were both approximately 10–15 mM (Fig. 4).

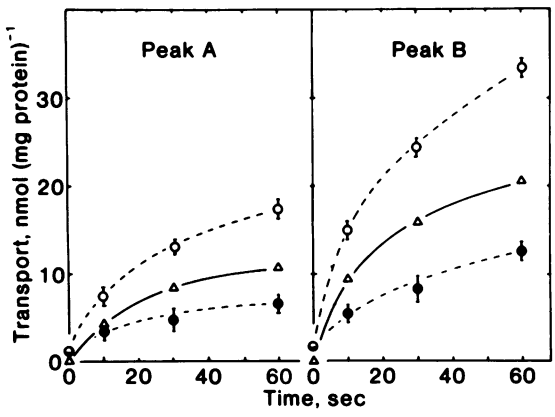

FIG. 3. Time courses of glucose uptake by liposomes reconstituted with the factors solubilized from either peak A or peak B of insulin-treated cells. Figure shows the total (○) and cytochalasin B-insensitive (●) transport activities as well as the difference (△) between the two. The points and short bars represent the mean values and the SEMs (n = 3–6).

DISCUSSION

Previously, Kasahara and Hinkle (5, 6) solubilized the glucose transport activity of erythrocyte ghosts and reconstituted it in liposomes. Similar work was done by Crane *et al.* (7) and Fairclough *et al.* (8) on the glucose transport activity of kidney, and by Shanahan and Czech (9) and Melchior and Czech (10) on the activity of fat cells. In our present study, we adapted their methods to estimate the glucose transport activity associated with subcellular structures. Our data (Figs. 2 and 3 and Table 1) suggest that such estimates can be made with reasonable precision. However, the accuracy of our data with respect to the relative activities in different subcellular fractions is debatable because little is known about the factors that may affect the solubilization and reconstitution of the transport activity. Therefore, the data showing the activities in different fractions must be compared with caution.

Prior to this study, we assumed that most of the glucose transport activity was associated with the plasma membrane, although a certain level of the activity had been detected in the lysosomal fraction (21). However, our present data indicate that the activity in fat cells is separable into two distinct fractions (peaks A and B) by sucrose density gradient centrifugation. Peak A in the gradient apparently coincides with a peak of protein that is associated with 5′-nucleotidase activity (Fig. 1). Previously, this peak was shown to coincide with the peaks of catecholamine-sensitive adenylate cyclase (17) and the receptors for insulin (1, 17), glucagon (1), and wheat germ agglutinin (1). Because all these activities are generally regarded as markers of the plasma membrane, we suggest that peak A represents the glucose transport activity in the plasma membrane.

Peak B in the sucrose gradient appears to coincide with the peak of UDPGal:*N*-acetylglucosamine galactosyltransferase (Fig. 1), a marker enzyme of the Golgi apparatus. It is possible

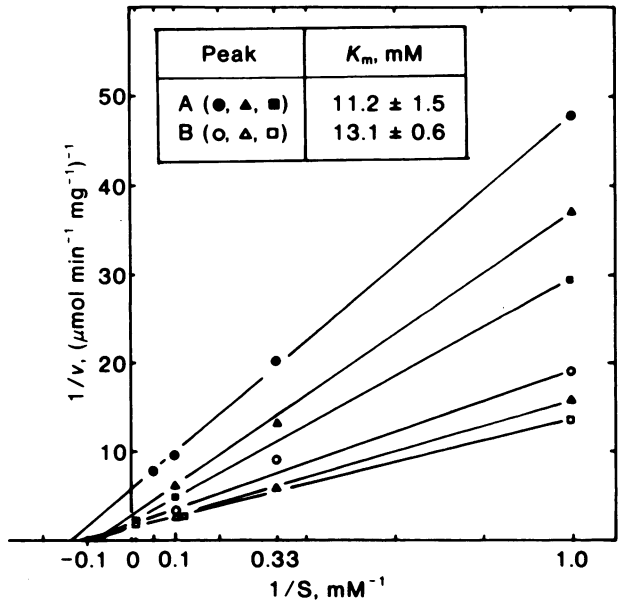

FIG. 4. Lineweaver–Burk plots of the glucose transport activities in liposomes reconstituted with the solubilized factors from peaks A and B of insulin-treated cells. The K_m values shown are the mean values ± SEM (n = 3).

that newly synthesized carriers for glucose transport are incorporated into the membranes of the Golgi apparatus, which are thought to become parts of the plasma membrane by exocytosis (22). It is also possible that the transport carriers are recycled between the plasma membrane and the Golgi apparatus by the processes suggested by Palade (22). However, it seems to be difficult to explain with these theories why peaks of other plasma membrane markers, such as 5′-nucleotidase, are not found in the Golgi fraction (Fig. 1 and refs. 1, 17, and 18). Furthermore, our present data do not necessarily indicate that the activity in peak B is physically associated with the Golgi apparatus. Therefore, the cellular site of activity in peak B is still an open question, although we can safely conclude that a considerable amount of the transport activity is associated with certain subcellular particles having sedimentation characteristics similar to or identical with those of the Golgi vesicles.

The effect of insulin on increasing the height of peak A (Fig. 1 and Table 1) might be expected because, as discussed above, this peak appears to represent the transport activity in the plasma membrane. It has been known that insulin at 1 nM, which is within the physiological concentration range of this hormone, stimulates the glucose transport activity of intact fat cells almost to the maximum in 5 min (4).

The effect of insulin on lowering the height of peak B cannot be explained with certainty. However, it can be postulated as a working hypothesis that peak B represents the transport activity in an intracellular storage site, and that insulin facilitates translocation of the activity from the storage site to the plasma membrane. Such translocation should increase the V_{max} value of glucose transport. It has been concluded earlier that insulin stimulates glucose transport in intact fat cells by increasing the V_{max} value rather than by changing the K_m (23). The K_m value of the glucose transport facilitated by the factors in peaks A and B are both approximately 10–15 mM (Fig. 4); this is consistent with the view that the transport activity is translocated. The affinity of glucose with its transport carrier of intact fat cells, determined as the K_i value by Loten *et al.* (24) at 37°C, is 13 mM; the same value, estimated by Whitesell and Gliemann (25) at 22°C, is 4 mM. In agreement with the translocation hypothesis, Wardzala *et al.* (26) have suggested that the effect of insulin might be to increase the number of the transport carriers, rather than to stimulate a fixed number of the carrier molecules. They found that the hormone increases the cytochalasin-binding sites in the plasma membrane. As mentioned earlier, cytochalasin B is a well-documented inhibitor of the carrier-mediated glucose transport (5, 6, 19, 20). The translocation hypothesis is also compatible with the observation that ATP or metabolic energy is needed for the action of insulin (18, 27, 28). If the translocation of the transport activity were brought about by exocytosis, the reaction might be coupled with the endocytotic internalization of cell-bound insulin, since the latter reaction also proceeds to a considerable extent in 5 min (1). It should be noted, however, that no direct evidence of the translocation has been obtained. It should also be mentioned that insulin increases the rate of glucose transport in intact fat cells about 5- to 10-fold (29). By contrast, the increase in the height of peak A observed in our present study is only approximately 2-fold (Fig. 1 and Table 1). Therefore, the proposed translocation of the transport activity might be a part of the mechanism by which insulin stimulates the cellular glucose transport under physiological conditions.

Note Added in Proof. Since the submission of our present report for publication, we have received a manuscript from Cushman and Wardzala (30). They found D-glucose-inhibitable cytochalasin B-binding activities in both plasma membrane and microsomal fractions. They further found that insulin increases the activity in the former fraction while decreasing the activity in the latter. Because cytochalasin B competitively binds to the glucose transport mechanism (26), their data seem to be consistent with our present observation.

We are indebted to Drs. S. P. Colowick and C. R. Park for valuable discussions and for reading this manuscript critically. We are thankful to Dr. C. C. Su for her helpful suggestions on reconstitution procedures. We are also grateful to Miss Lynn Dansey for her excellent technical assistance. This work was supported by Grants 5RO1 AM 06725 and 5RO1 AM 19925 from the National Institutes of Health.

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