Development of an *in vitro* reconstitution assay for glucose transporter 4 translocation

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In an attempt to define the mechanism of insulin-regulated glucose transporter 4 (Glut4) translocation, we have developed an *in vitro* reconstitution assay. Donor membranes from 3T3-L1 adipocytes transfected with mycGlut4 were incubated with plasma membrane (PM) from nontransfected 3T3-L1 cells, and the association was assessed by using two types of centrifugation assays. Association of mycGlut4 vesicles derived from donor membranes with the PM was concentration-, temperature-, time-, and Ca^{2+}-dependent but ATP-independent. Addition of a syntaxin 4 fusion protein produced a biphasic response, increasing association at low concentration and inhibiting association at higher concentrations. PM from insulin-stimulated cells showed an enhanced association as compared with those from untreated cells. Use of donor membranes from insulin-stimulated cells further enhanced the association and also enhanced association to the PM from isolated rat adipocytes. Addition of cytosol, GTP, or guanosine 5′-[γ-thio]triphosphate decreased the association. In summary, insulin-induced Glut4 translocation can be reconstituted *in vitro* to a limited extent by using isolated membranes. This association appears to involve protein–protein interactions among the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex proteins. Finally, the ability of insulin to enhance association depends on insulin-induced changes in the PM and, to a lesser extent, in the donor membranes.

One of the major physiological roles of insulin is a control of postprandial blood glucose, which occurs by stimulation of glucose uptake in skeletal muscles and adipocytes (1, 2). These two tissues, as well as cardiac muscle, express glucose transporter 4 (Glut4) and have the unique machinery required for the movement of Glut4 from intracellular pools to the plasma membrane (PM) (3–6). This translocation process is stimulated by insulin and accounts for a significant part of the insulin action in peripheral tissues for maintaining glucose homeostasis. Defects in insulin stimulation of Glut4 translocation also contributes to the insulin-resistance characteristic of type 2, non-insulin-dependent diabetes mellitus (7, 8).

Although the exact molecular and cellular mechanisms involved in insulin-stimulated Glut4 translocation remain unclear, this process requires activation of phosphatidylinositol 3-kinase (9, 10) and, possibly, serine/threonine phosphorylation events (11, 12). The vesicle-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) protein VAMP 2 (vesicle-associated membrane protein 2) and its homologue cellubrevin are associated with Glut4-containing vesicles (13, 14), suggesting that translocation of these vesicles uses machinery similar to that involved in vesicular trafficking in neuroendocrine tissues (15, 16). Indeed, cleavage of VAMP 2 and cellubrevin by botulinum neurotoxins or overexpression of mutant SNARE proteins is associated with an inhibition of insulin-stimulated Glut4 translocation (17–19). The target, t-SNARE complex protein, syntaxin 4, is present in the PM of adipocytes and also has been shown to be involved in Glut4 translocation (18, 20–23). Recently, a syntaxin 4-binding protein, Synip, was shown to be a regulatory component of the SNARE complex involved in Glut4 translocation (24). However, the exact nature of insulin signals that regulate Glut4 translocation and how they affect the donor membrane, PM, or some other component in the translocation machinery remain unclear.

In an attempt to address these questions, we have developed an *in vitro* reconstitution assay system for Glut4 translocation. Using this approach, we have attempted to define the site of insulin-induced effects on the association between the intracellular Glut4-containing vesicles and the PM and other factors that might contribute to the translocation process.

Experimental Procedures

Materials and Cell Lines. Insulin was purchased from Boehringer Mannheim. Polyclonal antiinsulin receptor antibodies and anti-IRS-1 antibodies were prepared as described (9). Monoclonal antiphosphotyrosine (4G10) and monoclonal anti-Myc (9E10) were purchased from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology, respectively. Polyclonal anti-Glut4 was purchased from East Acres Biologicals (Southbridge, MA). 3T3-L1 fibroblasts were transfected with a mycGlut4 cDNA by using retroviral expression plasmids as described (25). Stable transfectants were established after selection in the presence of puromycin (2 μg/ml) for 3 weeks. Resistant cells were screened further by immunoblotting for mycGlut4 expression, and successfully transfected cells were used for the experiments. Both transfected and nontransfected 3T3-L1 fibroblasts were differentiated by using isobutylmethylxanthine, dexamethasone, and insulin as described (26). The adipocytes were used 10 days after the initiation of differentiation.

Proteins or cDNAs. A soluble form of glutathione *S*-transferase (GST)-syntaxin 4, which lacks the C-terminal 25 aa encoding the transmembrane domain, was a kind gift of Richard H. Scheller (Stanford University Medical Center). A plasmid containing the cDNA for Glut4 with a myc-tag, P^{66}(AEEQKLISEEDLLK)G^{67}, was kindly provided by Christine Reynet (Novo-Nordisk, Copenhagen).

Preparation of Subcellular Fractions. Differentiated 3T3-L1 cells in 10-cm dishes were serum-starved for 18 h. Cells were incubated with or without 10^{-7} M insulin, washed twice with ice-cold PBS, and homogenized immediately by using 26 strokes of a 1-ml Teflon–glass homogenizer in a buffer containing 10 mM Hepes (pH 7.4), 1 mM EDTA, 255 mM sucrose, 1 mM DTT, 1 mM Na_3VO_4, 50 nM okadaic acid, 1 mM PMSF, and 0.1 mg/ml aprotinin. For preparing the donor membrane containing mycGlut4, the homogenization buffer was supplemented with 0.5 mM $ZnCl_2$ as an inhibitor of phosphatidylinositol phosphate phosphatases and phosphotyrosine phosphatases (27, 28). The

Abbreviations: Glut, glucose transporter; GST, glutathione *S*-transferase; PM, plasma membrane; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; GTP[γS], guanosine 5′-[γ-thio]triphosphate; IPM, insulin-stimulated PM; IDM, insulin-stimulated donor membrane; BPM, basal PM; BDM, basal donor membrane.

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homogenized cells then were subjected to subcellular fractionation as described previously to isolate PM and donor membranes (29, 30). The donor membrane fraction contained both low- and high-density microsomes. The resulting pellets of PM or donor membranes were suspended in the homogenization buffer without $ZnCl_2$. The protein concentration of both the PM and donor membranes was measured by using the Bradford method. Membrane fractions were used immediately.

***In Vitro* Association Assay.** After the subcellular fractionation procedure and normalization of protein concentration, donor membrane and PM (15–30 μg each) were mixed at 4°C in 75 μl of assay buffer (20 mM Hepes-KOH, pH 7.0/250 mM sucrose/0.5 mM EGTA/1.5 mM $MgCl_2$/0.5 mM $CaCl_2$/1 mM DTT/50 μg/ml BSA/50 mM KCl), supplemented with an ATP-regenerating system (1 mM ATP/8 mM creatine phosphate/25 units/ml creatine phosphokinase). The amount of protein for PM and donor membranes in the assay was equal, unless specified. The reactions were initiated by incubation at 37°C. After the indicated period, the reaction was returned to 4°C and subjected to one of two procedures to separate the PM or PM-associated donor membrane-derived vesicles.

Method I. The mixture was applied to the top of a discontinuous 28–44% sucrose gradient with 2% steps and subjected to centrifugation at 100,000 × *g* for 18 h. Each 4-ml step layer was diluted with ice-cold water and centrifuged at 200,000 × *g* for 1 h. The final pellets were dissolved in SDS-sample buffer and separated by SDS/PAGE.

Method II. The second method to reisolate the PM was a centrifugation at 15,000 × *g* for 15 min after dilution of the assay mixture with 1 ml of the ice-cold homogenization buffer supplemented with 0.5 M KCl. The final pellet was dissolved in SDS-sample buffer and separated by SDS/PAGE. For immunoblotting, proteins subjected to SDS/PAGE were electroblotted to nitrocellulose filters. The filters were blocked with 3% BSA and then incubated with the appropriate antibody, washed, reacted with anti-rabbit or anti-mouse IgG coupled to peroxidase, and developed with enhanced chemiluminescence reagents as instructed by the manufacturer (DuPont/NEN). The signal on the blot was quantitated by densitometry scanning. Using these methods, the signal for mycGlut4 on the blot was proportional to the load over the range used.

Results

Expression of mycGlut4 and Insulin-Induced Translocation. Fig. 1 shows a subcellular fractionation of 3T3-L1 adipocytes overexpressing mycGlut4. The PM, cytosol, and low-density microsomal membranes were analyzed by immunoblotting with either antiphosphotyrosine, anti-Glut4, or anti-myc. Insulin induced the tyrosine phosphorylation of multiple proteins, including the insulin receptor β-subunit at 95 kDa, the 46- and 52-kDa isoforms of Shc (most concentrated in the PM), IRS-1 (approximately equal in the intracellular membranes and the cytosol), and IRS-2 and mitogen-activated protein kinase, which were most abundant in the cytosol (Fig. 1*A*). Transfected mycGlut4 was detected as a broad band of 48–55 kDa, migrating slightly above endogenous Glut4 (Fig. 1 *B* and *C*). Total Glut4 content was about 2-fold higher in transfected cells than in nontransfected cells. Insulin induced the translocation of both mycGlut4 and endogenous Glut4 from the intracellular pool to the PM (Fig. 1 *B* and *C*), indicating that transfected mycGlut4 undergoes insulin-regulated translocation similar to endogenous Glut4 and, thus, provides a suitable method to study Glut4 translocation.

Reconstitution Assay. In an attempt to study the association between Glut4-containing vesicles and the PM, donor membranes prepared from mycGlut4 adipocytes were incubated with PM from nontransfected adipocytes, and the PM was reisolated and assayed for mycGlut4 content. Insulin-stimulated PMs were prepared from cells treated with insulin for 5 min, the approximate $t_{1/2}$ for appearance of Glut4 at the cell surface (31). Insulin-stimulated donor membranes were prepared from cells treated with insulin for 2.5 min. At this time, insulin-induced Glut4 translocation is not complete (there is an approximate 20% reduction of intracellular mycGlut4 content), and, thus, some insulin-responsive Glut4-containing vesicles should remain in the intracellular pool. Using equilibrium centrifugation with a discontinuous sucrose gradient, endogenous Glut4 in the PM was recovered in 34% and 36% sucrose fractions and Glut4 in the donor membrane was recovered in 38–42% fractions (Fig. 2*A*). As expected, no mycGlut4 was present in the PM of nontransfected cells (Fig. 2*B Top*), whereas it was easily detected in 38–42% sucrose fractions of the donor membrane of transfected cells, where it comigrated with endogenous Glut4 (compare *Middle* of Fig. 2 *A* and *B*). After incubation of the donor membrane from mycGlut4 cells with the PM from nontransfected cells, the mycGlut4 was shifted to the 36% and 38% sucrose fractions, indicating that some interaction between the PM and the intracellular mycGlut4-containing vesicles occurred *in vitro*. These data are quantified in Fig. 2*C*, which indicates more clearly the shift in position of the mycGlut4 in the mixed insulin-stimulated PM (IPM) and insulin-stimulated donor membrane (IDM) as compared with the IDM alone.

The gradient centrifugation technique indicated an association between the mycGlut4 vesicles and the PM. However, this method was cumbersome for processing numerous samples and difficult to quantify. Thus, a second method was developed for

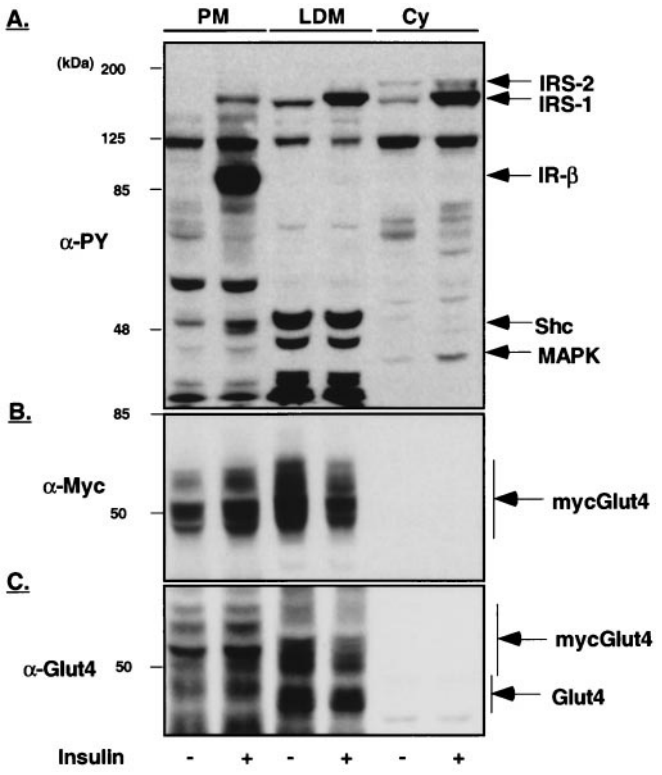

Fig. 1. Tyrosine phosphorylation and Glut4 translocation in mycGlut4-transfected 3T3-L1 adipocytes cells. 3T3-L1 adipocytes expressing mycGlut4 were stimulated with or without 10^{-7} M insulin for 10 min. Cells were harvested, homogenized, and subjected to subcellular fractionation as described in *Experimental Procedures*, and each fraction (PM, plasma membrane; LDM, low-density microsomes; Cy, cytosol) was subjected to SDS/PAGE and immunoblotting with antiphosphotyrosine (*A*), anti-myc (*B*), or anti-Glut4 (*C*).

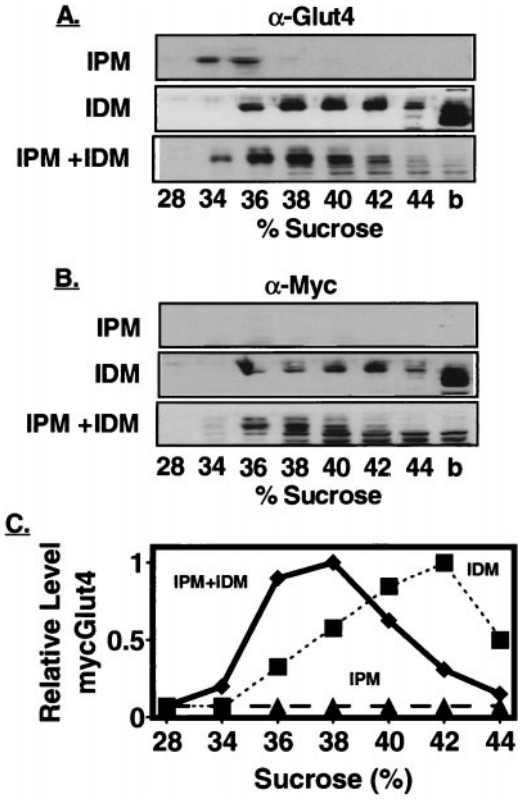

Fig. 2. Association assay using equilibrium centrifugation and a discontinuous sucrose gradient. The donor membranes (DM) were prepared from 3T3-L1 adipocytes expressing mycGlut4 treated with 10^{-7} M insulin for 2.5 min (IDM). The PM was prepared from nontransfected 3T3-L1 adipocytes treated with 10^{-7} M insulin for 5 min (IPM). The subcellular fractions (200 μg each) were incubated for 30 min at 37°C in a total volume of 500 μl (buffer included 20 mM Hepes-KOH (pH 7.0), 250 mM sucrose, 0.5 mM EGTA, 1.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM DTT, 50 μg/ml BSA, 50 mM KCl, and an ATP-regenerating system) and applied to the top of the sucrose gradient. Membrane pellets, prepared from the sucrose gradient fractions as described in *Experimental Procedures*, were separated by SDS/PAGE and analyzed by immunoblotting with anti-Glut4 (*A*) and anti-myc (*B*). The relative level of mycGlut4 in each fraction shown in *B* was quantitated by scanning densitometry, and the results are plotted in *C*.

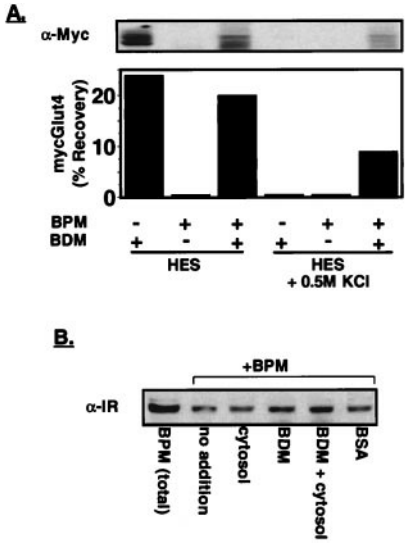

Fig. 3. Optimization of assay conditions I: dilution buffer and the recovery of membrane fractions. The donor membrane was prepared from 3T3-L1 adipocytes expressing mycGlut4. The PM and the cytosol were prepared from nontransfected 3T3-L1 adipocytes. The membrane preparations were incubated for 15 min at 37°C in a total volume of 75 μl [assay buffer included 20 mM Hepes-KOH (pH 7.0), 250 mM sucrose, 0.5 mM EGTA, 1.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM DTT, 50 μg/ml BSA, 50 mM KCl, and an ATP-regenerating system]. (*A*) The assay mixtures were diluted with homogenization buffer without (left three lanes) or with (right three lanes) 0.5 M KCl and then subjected to 15,000 $\times$ *g* centrifugation for the reisolation of the PM. The association between the PM and the intracellular mycGlut4-containing vesicles was analyzed by detection of mycGlut4 in the reisolated PM by anti-myc immunoblot (*Upper*). The immunoblots were quantified by scanning densitometry, and the data are expressed as a percentage of the total mycGlut4 included in the assay (*Lower*). (*B*) To test factors that might influence the recovery of PM, cytosol, BDM, or BSA was included during the incubation period and PM was recovered as described above. The recovery of the PM after reisolation was assessed by the recovery of the insulin receptor by immunoblotting with antibody to the β-subunit of the insulin receptor.

the reisolation of the PM by using centrifugation at 15,000 $\times$ *g* for 15 min after a 13-fold dilution of the sample. Under these conditions the PM pellets while the donor membranes remain in the supernatant. When the dilution was performed with ice-cold homogenization buffer, however, some mycGlut4 was observed in the absence of the PM because of nonspecific sticking of the donor membranes to the microfuge tubes (Fig. 3*A*). Addition of 0.5 M KCl to the dilution buffer eliminated this nonspecific interaction (Fig. 3*A*) and was, therefore, included in subsequent experiments. Under these conditions, about 50% of the PM was recovered in the final pellet, as judged by the recovery of the β-subunit of the insulin receptor (Fig. 3*B*); however, no donor membrane was recovered in the absence of PM.

Role of Divalent Cations and Nucleotides in Association. Although the exact mechanism of insulin-regulated translocation of Glut4 is unknown, this process shares similarities to regulated exocytosis in neuroendocrine cells. In these systems, Ca^{2+} plays an important role in triggering fusion (32). We examined the effect of several divalent cations on the association between the donor membrane-derived mycGlut4 and the PM of nontransfected cells. As shown in Fig. 4*A*, Ca^{2+} enhanced the association in a concentration-dependent manner with a 9-fold increase at 0.5 mM and a 14-fold increase at 5 mM. All subsequent experiments included 0.5 mM Ca^{2+} in the assay mixture. Mn^{2+} and Zn^{2+} at similar concentrations also enhanced the association (data not shown).

Generally, vesicular transport requires ATP and cytosolic proteins, and GTP is stimulatory in many systems of regulated exocytosis (32). However, in this system, omission of ATP from the assay did not alter the extent of the association, suggesting that the association is ATP-independent (Fig. 4*B*). Addition of 1 mM GTP decreased the association by 20–30% (Fig. 4*B*). A similar decrease also was observed by using guanosine 5′-[γ-thio]triphosphate (GTP[γS]) (data not shown). Addition of 100 nM wortmannin in the assay also had no effect on the association (Fig. 4*B*).

To further define the conditions of the assay, the PM dependency and time dependency of the association were examined. Increasing the amount of PM increased association almost linearly (Fig. 5*A*), i.e., the recovery of mycGlut4 in the final pellet depended on the presence and concentration of the PM. In addition, the association increased linearly with time up to 30 min at 37°C (Fig. 5*B*). No association was observed at 4°C over a 30-min period (data not shown).

Specificity of the *in Vitro* Interaction. To examine the specificity of the observed interaction between the donor membrane-derived mycGlut4 and the PM, we performed two sets of experiments.

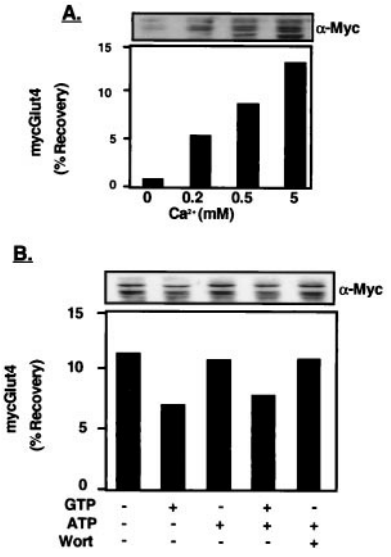

Fig. 4. Optimization of assay conditions II: divalent cation, ATP, or GTP dependence. The donor membrane was prepared from 3T3-L1 adipocytes expressing mycGlut4 treated with 10^{-7} M insulin for 2.5 min (IDM). The PM was prepared from nontransfected 3T3-L1 adipocytes treated with 10^{-7} M insulin for 5 min (IPM). The assays were performed as described in Fig. 3. (*A*) The assay was performed by using IPM and IDM in the presence of the indicated concentration of Ca^{2+}. Note that, in all cases, the assay mixture also included 0.5 mM EGTA. (*B*) The assay was performed in the presence and absence of 1 mM ATP, 1 mM GTP, or 100 nM wortmannin (Wort) as indicated.

Incubation of donor membranes prepared from nontransfected adipocytes with donor membranes from mycGlut4 cells effectively competed for association of the mycGlut4 with the PM. Incubation with BSA (0.8 mg/ml) or GST protein alone had no effect (Fig. 6). Numerous studies recently have established a functional role of SNARE-complex proteins in translocation of

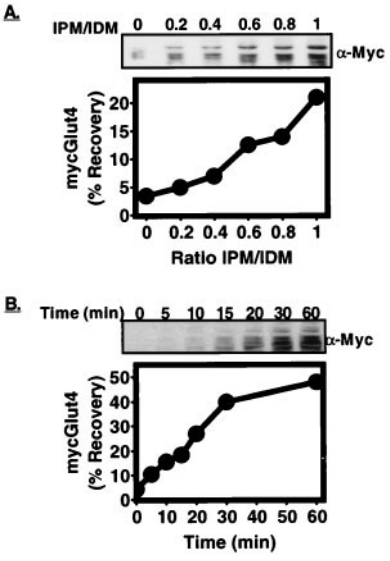

Fig. 5. The PM dependence and the time dependence of association. Donor membranes were prepared from 3T3-L1 adipocytes expressing mycGlut4 treated with 10^{-7} M insulin for 2.5 min (IDM). The PM was prepared from nontransfected 3T3-L1 adipocytes treated with 10^{-7} M insulin for 5 min (IPM). The assay shown in *A* was performed as described in Fig. 3 by using a fixed amount (30 μg) of IDM and increasing amounts of IPM (the ratio of IPM/IDM is indicated). (*B*) The assay was performed by using an equal amount (30 μg) of IPM and IDM for the indicated period of time.

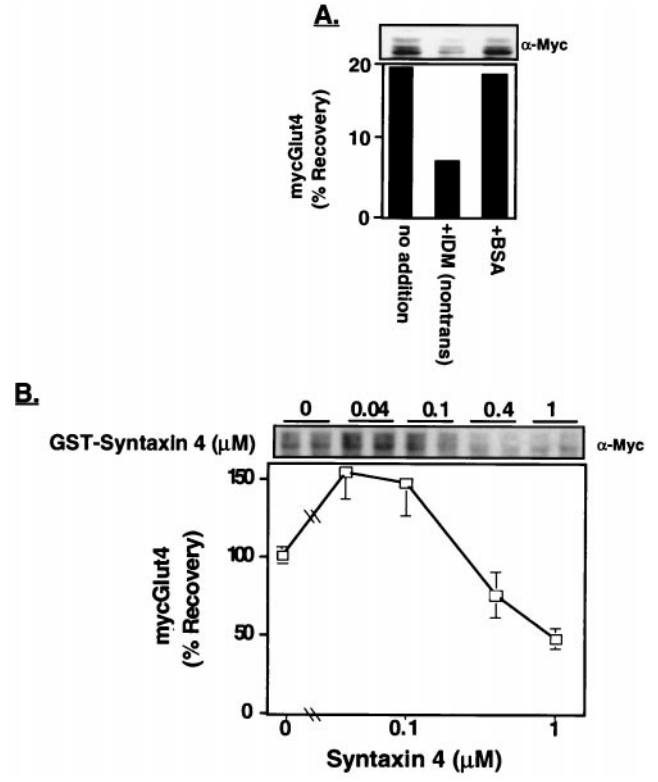

Fig. 6. Competitive inhibition of the *in vitro* association. The assay was performed by using 20 μg of IPM from nontransfected cells and/or IDM from mycGlut4 cells in the presence of 3-fold excess (0.8 mg/ml) of donor membrane from nontransfected adipocytes or BSA (*A*). (*B*) Assays were performed with IDM from mycGlut4 cells and IPM from nontransfected cells in the presence of the indicated concentration of GST-syntaxin 4. The total added protein concentration was normalized by the addition of GST protein. The quantified values of association as measured by mycGlut4 immunoreactivity are expressed as a percentage of the value without GST-syntaxin 4 ($n = 4$).

Glut4 vesicles. Indeed, disruption of v- and t-SNARE complex formation by cleavage with botulinum neurotoxins or overexpression of mutant SNARE proteins leads to inhibition of insulin-stimulated Glut4 translocation (17–19). Therefore, we examined the effects of GST-syntaxin 4 on the *in vitro* association of the donor mycGlut4 vesicles with the PM. Interestingly, at low concentrations (less than 0.1 μM), GST-syntaxin 4 enhanced the association of Glut4 vesicles with the PM by approximately 50%, whereas at higher concentrations, GST-syntaxin 4 inhibited the association by 50% (Fig. 6*B*).

Insulin-Induced Changes in the Donor and Acceptor Membrane Compartments. A fundamental question in insulin stimulation of glucose transport is whether the stimulation event occurs at the level of the intracellular vesicle, the PM, or some intermediate compartment. As shown in Fig. 7, using PM from control cells, there was no significant difference in the extent of association with mycGlut4-containing donor membranes isolated from either insulin-stimulated or nonstimulated cells [basal PM (BPM) + basal donor membrane (BDM); 8.5 ± 1.8% vs. BPM + IDM; 9.6 ± 2.6%]. By contrast, mycGlut4-containing membranes associated with the PM from insulin-stimulated cells to a significantly greater extent than that from control cells (BPM + BDM; 8.5 ± 1.8% vs. IPM + BDM; 13.4 ± 1.7%). The association was increased further if donor membranes from insulin-stimulated cells were incubated with insulin-stimulated PM (IPM + BDM; 13.4 ± 1.7% vs. IPM + IDM; 18.8 ± 3.1%). Thus, insulin enhanced the association 2.2-fold when comparing

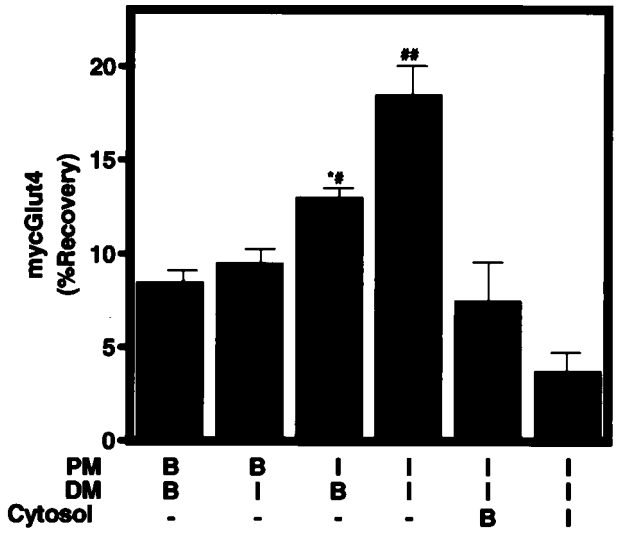

Fig. 7. Effect of insulin on the membrane components and cytosol. Donor membranes were prepared from 3T3-L1 adipocytes expressing mycGlut4 treated with or without 10^{-7} M insulin for 2.5 min. The PM and the cytosol were prepared from nontransfected 3T3-L1 adipocytes treated with or without 10^{-7} M insulin for 5 min (B, basal; I, insulin-stimulated). Reactions were performed as in Fig. 3. Results are expressed as the percentage of total mycGlut4 in the assay ($n = 5$, means ± SEM). Statistical significance was determined for pairwise comparisons within each experiment: *, $P < 0.01$ compared with BPM + BDM; #, $P < 0.05$ compared with BPM + IDM; and ##, $P < 0.05$ compared with BPM + BDM, BPM + IDM, and IPM + BDM.

membranes derived from basal cells vs. those prepared from insulin-treated cells. The insulin-induced changes in the PM responsible for the enhancement occurred between 1 and 4.5 min after the insulin addition and increased further with time (not shown). Insulin stimulation of both the PM and donor membrane compartments appears to be necessary for the maximal association, but some insulin-induced changes can be observed using only the PM from insulin-stimulated cells (similar results were observed with membranes from rat adipocytes). Incubation of cytosol (0.8 mg/ml) isolated from control cells with PM and donor membrane from insulin-treated cells resulted in a complete block in the insulin-enhanced association. Using cytosol from insulin-treated cells resulted in an even further inhibition of association: below levels observed in assays using PM and donor membrane from control cells (Fig. 7).

Discussion

The exact mechanism of translocation and the subcellular location(s) of insulin action in Glut4 translocation remain largely unknown. To help address these issues, we have developed an *in vitro* reconstitution assay by using principles developed for other *in vitro* membrane-trafficking systems (33, 34). To distinguish Glut4 molecules from the donor and recipient membrane, we used 3T3-L1 adipocytes expressing mycGlut4 as a source of donor membranes. When donor membranes containing mycGlut4 and PM were mixed, almost no mycGlut4 was recovered with the PM in the absence of divalent cations. In the presence of divalent cation (Ca^{2+} or Mg^{2+}), the association between mycGlut4 and the PM increased, suggesting that some divalent cation-induced change in either membrane fraction is important for the association. Although insulin causes only small changes in intracellular Ca^{2+} concentration (35), increasing or decreasing Ca^{2+} concentration inhibits insulin stimulation of glucose transport (36), suggesting that some optimal range of Ca^{2+} concentration may be required. Also, both Ca^{2+} and Mg^{2+} have been shown to stabilize lipid bilayers and induce membrane fusion (37, 38). This stabilization could result in more efficient protein–protein interaction and, thus, enhance association between membrane fractions.

The observed association depended on the amount of the PM and was time- and temperature-dependent. More importantly, the association can be inhibited competitively by a donor membrane fraction from nontransfected cells and by GST-syntaxin 4. BSA or GST alone had no effect. These data provide evidence for the specificity of the *in vitro* association.

Results from this study have provided some interesting observations related to the question of which subcellular compartment(s) are affected by insulin, resulting in increased Glut4 translocation. Clearly, the PM from insulin-stimulated cells had an increased capacity to associate with mycGlut4 vesicles as compared with those from nonstimulated cells, indicating that insulin-induced enhancement of the association mainly was the results of changes in the PM. The insulin-induced change in the PM occurred between 1 and 4.5 min after insulin addition, consistent with the time course of insulin-stimulated glucose transport. Using PM from insulin-stimulated 3T3-L1 adipocytes or freshly isolated rat adipocytes, mycGlut4 in donor membranes from insulin-stimulated cells also showed a higher level of association than those from nonstimulated cells. Therefore, maximum association requires insulin-induced changes in both the donor membrane and the PM compartments.

Several unexpected observations were made during the course of this study. One is the ATP-independent nature of the *in vitro* association between the mycGlut4-donor membranes and the PM. Clearly, in intact and permeablized (39) cells, insulin action in Glut4 translocation is ATP-dependent. In mast cells, regulated exocytosis occurs after a lag period of a minute or so (40) and appears to be ATP-independent (41). Insulin-induced Glut4 translocation is also a relatively slow process compared with stimulated exocytosis in neuroendocrine cells. In addition, GTP[γS]-stimulated Glut4 translocation is ATP-independent in permeabilized 3T3-L1 adipocytes (39). Moreover, the lack of ATP dependence is not too surprising, because the data strongly suggest that the observed *in vitro* association is directed by specific interactions between the v- and t-SNARE proteins that have been shown to form highly stable, SDS-resistant complexes in an ATP-independent manner (15, 16).

Another unexpected observation was the effect of cytosol. Addition of cytosol caused a decreased in the *in vitro* association of mycGlut4 with the PM. This inhibitory activity was enhanced in cytosol prepared from insulin-treated cells. This was an unexpected result because cytosol is generally necessary for the process of vesicular transport in other *in vitro* assays (32). One important factor required for many vesicle-trafficking functions is *N*-ethylmaleimide-sensitive fusion protein (NSF). NSF may be important for an ATP-dependent priming step, i.e., producing a latent, fusogenically active state, rather than allowing for actual docking of the intracellular vesicles to the PM as measured in this assay (42). NSF and α-SNAP are associated with Glut4-containing intracellular vesicles in rat adipocytes (43). Thus, in the present assay system, cytosol (and ATP) may not be necessary, because priming of the vesicles may have already occurred in the cells, allowing us to observe an enhancement of *in vitro* association after the *in vivo* insulin stimulation in the PM and donor membrane compartments.

In permeabilized adipocytes, GTP[γS] has been reported to induce Glut4 translocation (39, 44) and stimulate other regulated exocytotic systems (32). In this *in vitro* assay, GTP or GTP[γS] caused a modest decrease in membrane association. This may reflect the effect of GTP analogues to inhibit the reconstituted glucose transporter activity as reported by Schürmann *et al.* (45). GTP[γS] also has been shown to block constitutive exocytosis (32). Thus, the effect of GTP analogues to stimulate Glut4 translocation in permeabilized cells may depend on modulating

some other component of the signaling pathway. Of course, the findings that cytosol and the guanine nucleotides had somewhat inhibitory effects also may indicate limitations in the *in vitro* assay system. For example, it is possible that cytosol and GTP act at some bridge between the PM and the Glut4-containing vesicles and that the cytosol and GTP would enhance association if additional regulatory proteins were included in the *in vitro* assay. Further characterization of the cytosol and guanine nucleotide effects will be required.

In summary, these data establish an *in vitro* assay system for Glut4 translocation and succeed in recreating some of the insulin-induced effects at both the PM and the donor membrane compartments. Refinement of this system should help elucidate some of the fundamental biochemical aspects of Glut4 translocation.

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