GLUT4-containing vesicles are released from membranes by phospholipase D cleavage of a GPI anchor

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Kristiansen, Søren, and Erik A. Richter. GLUT4-containing vesicles are released from membranes by phospholipase D cleavage of a GPI anchor. Am J Physiol Endocrinol Metab 283: E374–E382, 2002.—We have previously developed a cell-free assay from rat skeletal muscle that displayed in vitro glucose transporter 4 (GLUT4) transfer from large to small membrane structures by the addition of a cytosolic protein fraction. By combining protein fractionation and the in vitro GLUT4 transfer assay, we have purified a glycosylphosphatidylinositol (GPI) phospholipase D (PLD) that induces transfer of GLUT4 from small to large membranes. The in vitro GLUT4 transfer was activated and inhibited by suramin and 1,10-phenanthroline (an activator and an inhibitor of GPI-PLD activity, respectively). Furthermore, upon purification of the GLUT4 transporter protein, the protein displayed an elution profile in which the molecular mass was related to the charge, suggesting the presence or absence of phosphate. Second, by photoaffinity labeling of the purified GLUT4 with 3-(trifluoromethyl)-3-(m-[125I]iodopropyl)diazirine, both labeled phosphatidylethanolamine and fatty acids (constituents of a GPI link) were recovered. Third, by using phase transition of Triton X-114, the purified GLUT4 was found to be partly detergent resistant, which is a known characteristic of GPI-linked proteins. Fourth, the purified GLUT4 protein was recognized by an antibody raised specifically against GPI links. In conclusion, GLUT4-containing vesicles may be released from a membrane compartment by action of a GPI-PLD.

insulin signaling; glucose metabolism; rat skeletal muscle; glucose transporter 4; glycosylphosphatidylinositol

THE INSULIN AND MUSCLE contraction-responsive glucose transporter 4 (GLUT4)-containing vesicles are localized to tubulovesicular elements in the basal state (28). Upon stimulation, the GLUT4-containing vesicles undergo translocation to the surface membrane. Despite extensive scrutiny, the upstream insulin- and contraction-mediated signaling proteins leading to GLUT4 translocation are not described in great detail. We have previously focused on immunoprecipitation of low-spin membranes containing GLUT4 protein and looked for other protein(s) associated within the membrane compartment (18). Using this approach, we found that the GLUT4 protein was associated with an insulin-insensitive phosphatidylinositol (PI) 4-kinase. Furthermore, in a recently published paper (17), we also reported that in vivo insulin stimulation resulted in an increased production of phosphatidic acid in the GLUT4 immunoprecipitate. Because phosphatidic acid is a known product of phospholipase D (PLD) action, this suggested to us that a PLD could be part of the mechanism leading to GLUT4 mobilization. For a number of reasons, we initially hypothesized that it could be the ADP ribosylation factor (ARF)-sensitive PLD1 (14). Because of the restricted experimental models revealing ongoing enzymatic reactions in skeletal muscle, we were prompted to develop an in vitro assay to investigate whether ARF1 or 6 and subsequent PLD1 activation could lead to GLUT4 translocation (17), which was also suggested by Emoto et al. (9). In brief, we reported that adding a cytosolic protein fraction (which may contain upstream signaling molecules such as ARFs) to membranes containing GLUT4 resulted in a transfer of GLUT4 proteins from large to small membrane structures. This transfer could be inhibited by neomycin (a PLD inhibitor). Nevertheless, we were not able to replace the unknown cytosolic molecule(s) by adding recombinant myristoylated ARF1 or ARF6. Also, ARF1 inhibitory peptide did not block the GLUT4 transfer. Taken together, the previous data (17) suggested that PLD could indeed be involved in the GLUT4 mobilization process, but it was probably not an ARF-sensitive PLD1. We have now taken advantage of the in vitro assay, and by protein fractionation we have purified a protein fraction that induces in vitro GLUT4 translocation. This fractionation procedure resulted in copurification of a PLD that is known to cleave glycosylphosphatidylinositol (GPI) anchors (7, 15).

METHODS

We took advantage of the partial in vitro reconstitution of the GLUT4 translocation mechanism (17). In brief, an enriched GLUT4 membrane fraction (~120 μg of protein) was isolated by density gradient centrifugation of a postnuclear supernatant. This enriched GLUT4 fraction was added con-
trol buffer or a cytosolic protein fraction (~225 µg of protein; see next section). Small and large membrane structures were then separated by differential centrifugation. The GLUT4 protein content was determined in the supernatant containing the small membrane structures and in the pellet containing larger membrane structures.

Method for isolation of the unknown cytosolic protein. It was found that the unknown cytosolic factor was present in large amounts in human blood. Thus 40 ml of plasma proteins were precipitated with polyethylene glycol (9% wt/vol), and to the supernatant (10 min, 4,000 g, 4°C) an equal volume of 250 mM bis-Tris, pH 6.35, was added. All chromatographic steps were carried out with a Biologic HR workstation (Bio-Rad, Hercules, CA) operating at 5°C. The purification process was monitored and controlled with a Biologic HR software system. First, the solution was run (flow 3 ml/min) through a column (2.5 cm × 20 cm) containing Q Sepharose fast flow (Amersham Pharmacia Biotech, Buckinghamshire, UK). Noncaptured proteins were washed out of the column with 150 ml of 50 mM bis-Tris and 10 mM NaCl, pH 6.35. Bound material was eluted with a linear increase to a final concentration of 500 mM NaCl. Active fractions eluted in a broad peak ~30 mS/cm. Solid NaCl was added to a final concentration of 500 mM to the active fractions, which were then subjected to immobilized metal affinity chromatography (IMAC). A column (1.5 cm × 5 cm) prepacked with iminodiacetic acid (Pierce, Rockford, IL) was activated with 500 mM zinc acetate and equilibrated with 50 mM Tris and 500 mM NaCl, pH 7.5 (flow 1 ml/min). The sample was run through the column and washed with equilibration buffer. Captured proteins were eluted with equilibration buffer containing 10 mM histidine. Active fractions were then separated by size-exclusion chromatography (Sephacryl S-200 HR) on a 2.5 cm × 100 cm column (flow 1 ml/min) using equilibration buffer. The GLUT4-mobilizing activity eluted in one to two fractions. Proteins in the active and neighboring fractions were then separated by SDS-PAGE (7.5%). The gel was silver stained using a kit (Amersham Pharmacia Biotech) to visualize all proteins.

Isolation of the GLUT4 protein. Approximately 20 g of fresh unfrozen rat skeletal muscle were transferred to 100 ml of homogenization buffer (50 mM Tris base, 25 mM NaCl, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.4) and blended for 1 min. The homogenate was centrifuged (10 min, 1,500 g, 4°C), and the resulting supernatant was centrifuged (30 min, 230,000 g, 4°C). The membrane pellet was resuspended in 30 ml of ice-cold strip buffer (100 mM NaCl, pH 10.0) and repelleted. This pellet was resuspended in solubilization buffer (50 mM MES, 50 mM bis-Tris, 25 mM NaCl, and 0.2% (vol/vol) Triton X-114, pH 5.5) to a final protein concentration of 0.20 mg/ml. The samples were left on ice for 30 min with agitation. The samples were centrifuged (30 min, 100,000 g, 4°C) and the supernatant was run (flow 3.0 ml/min) through a chromatography column (2.5 cm × 20 cm) containing Q Sepharose fast flow (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated in solubilization buffer. The column was washed with 100 ml of solubilization buffer. The GLUT4 protein was eluted with a linear increase in the NaCl concentration (25 mM-1 M) in solubilization buffer. Fractions containing GLUT4 were dissolved in an equal volume of 5 mM sodium phosphate buffer, pH 6.8, containing 0.2% (vol/vol) Triton X-114 and subjected to a column containing ceramic hydroxyapatite (Bio-Rad). Bound GLUT4 was eluted with a linear increase in sodium phosphate (5–200 mM) containing 0.2% Triton X-114. Fractions containing GLUT4 were captured on a Mono Q (5/5) column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris, 10 mM NaCl, and 0.2% (vol/vol) Triton X-114, pH 7.4. The GLUT4 protein was eluted by a linear increase in the NaCl concentration (10–500 mM). Based on silver staining, the fractions contained exclusively the GLUT4 protein. If the GLUT4 protein was prepared for 3-(trifluoromethyl)-3-(m-125I)iodophenyl)diazirine ([125I]TID) photoaffinity labeling, detergent was removed from the Mono Q column by extensively washing with sodium phosphate buffer, pH 8.5, before the GLUT4 protein was eluted with a linear increase (0–1 M) in NaCl dissolved in sodium phosphate buffer.

Photoaffinity labeling of GLUT4. The GLUT4 was upconcentrated by centrifugation through an Amicon 10 filter (Amicon). [125I]TID (10 µCi; Amersham Pharmacia Biotech) was added to the sample, which was photoaffinity labeled for 30 min at 350 nm (14a, 29). For the detection of phospholipids, the GLUT4 sample was loaded on a chromatography column (1.5 cm × 5.0 cm) containing Q Sepharose fast flow equilibrated in 5 mM sodium phosphate, pH 5.5. The loaded GLUT4 was incubated with the column and most of the labeled TID was washed out of the column by 50 ml of buffer and at a flow of 1 ml/min operated with a P1 pump device (Amersham Pharmacia Biotech). Fractions of 1 ml were collected by elution with 5 mM sodium phosphate, pH 8.5, containing 1 M NaCl. Five microliters of 6 M HCl and 200 mM sodium nitrite were added to all fractions, which were subsequently incubated at 60°C for 4 h. Positive GLUT4 and negative control fractions were added to 500 µl of chloroform-methanol (2:1), followed by centrifugation. The chloroform phase was collected, and thin-layer chromatography was performed on 20 × 20-cm silica gel plates (Sigma) developed in a solvent of chloroform-methanol-water (65:25:4). For the detection of neutral lipids, the photoaffinity-labeled GLUT4 was dried under a stream of nitrogen to remove the volatile unlabeled TID. Two hundred microliters of 0.1 M NaOH were added to the sample, which was then incubated overnight at 4°C, and then 500 µl chloroform-methanol (2:1) and acetic acid were added to neutralize the NaOH. The chloroform phase was loaded on a thin-layer chromatography (TLC) plate and developed in solvent of hexane-diethylether-acetic acid (60:30:1). The developed plate was dried, and radioactive components were detected with a STORM Phospholmager (Molecular Dynamics). The positions of phospholipid standards were detected by submerging the TLC plate in 10% CuSO4 and 8% phosphoric acid and baking the plate for 1 h at 100°C. The palmitic acid standard was detected by exposure of dried plates to iodine vapor.

SDS-PAGE and Western blotting. Proteins were separated on SDS polyacrylamide gels. Some gels were silver stained using a commercial kit (Amersham Pharmacia Biotech), or proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were Ponceaus S stained, destained, and blocked with 5% (wt/vol) BSA in TS buffer [50 mM Tris, 150 mM NaCl, 0.05% (vol/vol) Nonidet P-40, and 0.05% (vol/vol) Tween 20, pH 7.4] for 1.5 h at room temperature. The PVDF membranes were then incubated with a primary antibody dissolved in TS buffer containing 1% (wt/vol) BSA and 0.02% NaN3 (the antibodies were a goat anti-GLUT4 IgG, a rabbit anti-cross-reacting determinant (CRD) IgG, or a mouse monoclonal anti-GPI-PLD IgG purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Glyko (Novato, CA), or Transduction Laboratories (Lexington, KY), respectively). Antigen and primary antibody complexes were visualized upon binding with a secondary antibody conjugated with alkaline phosphatase (DAKO, Glostrup, Copenhagen, Denmark) and an enhanced chemilumines-
cence kit (Amersham Pharmacia Biotech). Fluorescence was detected with a STORM phosphoimager (Molecular Dynamics).

Statistics. All results are shown as mean values ± SE with a significance level at \( P < 0.05 \). Paired and unpaired \( t \)-tests were used to calculate the \( P \) level.

RESULTS

In this study, we have used a previously developed assay that displays in vitro GLUT4 transfer from large to small membrane structures (17). In brief, the assay is performed by incubation of a mixture of an enriched GLUT4 membrane fraction and cytosolic proteins. The GLUT4 protein content is then determined in the fast-pelleting large membrane fraction and in the slow-pelleting small membrane fraction, as shown in Fig. 1, top. In particular, the increase in the GLUT4 protein content in the small membrane fraction is not caused by adding GLUT4 protein contained in the cytosol, since no GLUT4 signal could be detected in the supernatant and pellet in experiments in which cytosol was added alone.

The in vitro GLUT4 assay is dependent upon the prior in vivo physiological condition of the rat. In brief, by adding unfrozen insulin-stimulated (1 min) or basal cytosol, there is an increase in GLUT4 transfer from large to small membrane structures in an enriched GLUT4 fraction isolated from a basal skeletal muscle (Fig. 1, middle). However, unfrozen cytosol from insulin-stimulated unfrozen skeletal muscle was only \( \sim 10–20\% \) more potent compared with unfrozen basal cytosol. This order of potency between basal and insulin-stimulated cytosol is diminished with frozen cytosol (data not shown). In particular, adding basal or insulin-stimulated (1 min) cytosol to an enriched GLUT4 membrane fraction from prior insulin-stimulated (10 min) skeletal muscle was not as potent as using basal skeletal muscle as a source of the enriched GLUT4 donor membrane fraction (Fig. 1, middle). We tested whether cytosolic protein fractions prepared from other tissue sources (liver, brain, blood plasma) than rat skeletal muscle could induce in vitro GLUT4 transfer. We found that blood plasma and to a lesser extent brain very potently induced in vitro GLUT4 transfer, as shown in Fig. 1, bottom. Thus blood plasma seemed to be a good source of the unknown stimulating factor, and we therefore set out to identify the unknown stimulating factor found in the plasma.

Subsequently, by protein chromatography of human plasma, we identified elute fractions that were able to induce in vitro GLUT4 transfer. First, to the resulting supernatant from polyethylene glycol precipitation, 50 mM MES, bis-Tris, or Tris buffer was added to give different \( \mathrm{pH} \) values (5.8, 6.3, and 7.8, respectively). The solutions were subjected to anion and cation exchange. In brief, it was found that the unknown factor did bind to a cation resin at \( \mathrm{pH} \) 5.8 but not at 6.3 and 7.8. The factor did also bind to anion resin at \( \mathrm{pH} \) 7.8 and at 6.3 with a lower affinity, but not at 5.8 (data not shown). This suggested that the isoelectric point for the protein(s) was between 5.8 and 6.3. We then performed anion exchange at \( \mathrm{pH} \) 6.35. The procedure for identification of the unknown protein is outlined in Fig. 2. Elute fractions able to induce GLUT4 transfer in the in vitro assay were then collected. Because we have previously observed that the in vitro GLUT4 transfer is sensitive to the concentration of divalent cations, we performed metal affinity chromatography. Indeed, the fractions that eluted with the chelating amino acid histidine were able to induce GLUT4 transfer. These fractions were then protein fractionated using a third parameter, namely size-exclusion chromatography. Interestingly, only one to two elute fraction(s) were able to induce GLUT4 transfer. The elution profile suggested that the native protein had a molecular size higher than the range of 200–250 kDa. The active one to two fraction(s) and the neighboring nonactive fractions were then electrophoresed on a 7.5% denaturing
gel, as shown in Fig. 2. All proteins were detected by silver staining to show the presence of an extra band in the active fractions compared with the nonactive fractions. Only one extra band (H11011 110 kDa) appeared in the active fractions, and this extra band was identified by immunoblotting to be GPI-PLD (Fig. 2).

Based on our observations regarding the source for purification (plasma), isoelectric point (5.8–6.3), presence of histidine in the protein, and the molecular mass of the native and denatured protein (200–250 kDa and 110 kDa), and our previous observation of production of phosphatidic acid in GLUT4 immunoprecipitates, we hypothesized that the unknown protein could be GPI-PLD (20, 30). This was tested with a GPI-PLD-specific antibody. Indeed, GPI-PLD eluted exclusively in the active fraction(s) able to induce GLUT4 transfer, and the immunoblot signal appeared at the same molecular mass as the extra band in the silver stain (Fig. 2, bottom).

This suggested that the GLUT4-containing vesicles could be linked to a large membrane compartment via a GPI link (see Fig. 8). We wanted to verify this hypothesis. First, we added 10 mM of suramin and 1 mM of 1,10-phenanthroline [an activator (21) and inhibitor (24) of membrane-bound GPI-PLD activity, respectively] to the GLUT4 in vitro assay. As shown in Fig. 3, top, suramin was able to potentiate the effect of plasma. Interestingly, suramin also potentiated the GLUT4 transfer in the absence of plasma proteins, suggesting that some of the GPI-PLD could be associated with the large membrane compartment. Finally, 1 mM 1,10-phenanthroline did inhibit the in vitro GLUT4 transfer (Fig. 3, bottom).

Furthermore, we wanted to purify the GLUT4 protein to chemically verify the copurification of a GPI link. The procedure for purifying the GLUT4 protein is outlined in Fig. 4. The initial source was a rat skeletal muscle homogenate. Next, a membrane preparation was obtained by centrifugation, and peripheral membrane proteins were stripped off. The integral mem-
brane proteins were solubilized and purified by anion-exchange and hydroxyapatite chromatography. The GLUT4 transporter was >99% pure based on silver staining of proteins in a 10% gel loaded with a muscle homogenate (lane 1), a fraction containing peripheral and integral membrane proteins (lane 2), integral proteins (lane 3), positive GLUT4 elute fractions from Q Sepharose (lane 4), hydroxyapatite (lane 5), a Mono Q chromatography (lane 6), and a molecular mass marker (lane 7). A clear radioactive signal appeared in the positive GLUT4 fractions. No signal was detected in labeled control fractions devoid of GLUT4, suggesting that the signal originated from the GLUT4 protein. The signal had a migration pattern similar to phosphatidylethanolamine, a known constituent in a GPI link. Furthermore, the GLUT4 protein did bind to the positive anion resin at pH 5.5, which is a much lower pH value than the calculated isoelectric point based on the polypeptide sequence. Thus an extra negative charge must be bound directly to the GLUT4 protein. In fact, when the elute fraction from the final chromatography Mono Q step was separated on a gel and silver stained, the GLUT4 protein eluted in three bands at ~50 kDa (Fig. 5). All three bands were recognized by the anti-GLUT4 IgG. The triplets were not uniformly distributed in the elute fractions since the high-molecular GLUT4 eluted before the low-molecular GLUT4 isotype. The high-molecular isotype may then have a higher isoelectric point (carrying relatively more positive charges at the same pH), which causes elution before the relatively more negative low-molecular isoform. This could be explained by the presence (noncleaved) or absence (cleaved) of a negatively charged phosphate molecule as part of a GPI link (see Fig. 8 in DISCUSSION). Furthermore, it may also explain why the GLUT4 protein could bind to the positive anion resin at a lower pH value than predicted from the calculated isoelectric point. The calculated isoelectric point is exclusively based on the polypeptide sequence and does not include conjugated groups.

Next, we wanted to see whether we could recover constituents from a GPI link by labeling of all of the reactive groups found on the isolated GLUT4 protein. In brief, the GLUT4 protein was captured on a chromatography column that was extensively washed with detergent to remove all lipids. The GLUT4 protein was then eluted without detergent, and the purified GLUT4 transporter and its reactive chemical site groups were photoaffinity labeled with \(^{125}\text{I}\)TID. In experiments designed for detection of phospholipids, the labeled GLUT4 protein was again captured on an anion-exchange column, and the excess of free unlabeled TID was washed away. The GLUT4 protein was then eluted from the column, and all elute fractions were nitrous deaminated. Fractions containing GLUT4 were then identified. Lipids were then extracted from fractions with GLUT4 and from fractions devoid of GLUT4 and separated on a TLC plate, as shown in Fig. 6. A clear radioactive signal appeared in the positive GLUT4 fractions. No signal was detected in labeled control fractions devoid of GLUT4, suggesting that the signal originated from the GLUT4 protein. The signal had a migration pattern similar to phosphatidylethanolamine, a known constituent in a GPI link. Furthermore, for the detection of fatty acids, the photoaffinity-labeled GLUT4 was hydrolyzed before any possible lipids were extracted and separated on a TLC plate. Interestingly, now two radioactive bands appeared with a migration pattern similar to the palmitic acid standard.

Furthermore, we took advantage of the isolation of GLUT4 protein in the presence of the detergent Triton X-114. Logically, the GLUT4 protein was detected in a Western blot in the water-detergent solution, as shown in Fig. 7, top. By phase transition and separation of water from pure detergent, proteins could be separated into the detergent phase and water phase. The GLUT4 could be immunodetected in the detergent phase, but hardly any signal was detected from the water phase. It should be noted that separation of detergent from the water phase results in a large volume of water and a minor volume of detergent. However, only a fraction of the water volume is run on the gel because of the size of the well in the gel. Thus the GLUT4 content in the
water phase is higher than indicated in Fig. 7. When the protein was upconcentrated by ammonium sulfate precipitation, the protein in the water phase did in fact react with a GLUT4-specific antibody, as shown in Fig. 7, bottom. This strongly suggest the presence of GLUT4 in the water phase. This is a known behavior for GPI-linked proteins.

Finally, we separated the pure GLUT4 transporter on a SDS gel and immunoblotted with a CRD antibody raised against an epitope that is uniquely formed on GPI proteins treated with PI phospholipase C (4, 16). The CRD antibody reacted against a positive standard prepared from the variant surface glycoprotein prepared by Trypanosoma brucei. Interestingly, this antibody also cross-reacted with the GLUT4 transporter, as shown in Fig. 8.
DISCUSSION

Here we report on the isolation of a GPI-PLD, which may be able to release GLUT4-containing vesicles from membranes by cleavage of a GPI link on the GLUT4 transporter. Furthermore, we purified the GLUT4 protein and provided immunological and biochemical evidence for the novel concept that GLUT4 may indeed contain such a GPI link (Fig. 9).

We have previously published evidence that a PLD may be involved in the mobilization of GLUT4-containing vesicles in skeletal muscle (17). This was primarily based on the finding that in vivo insulin stimulation resulted in an increased production of phosphatidic acid in the GLUT4 immunoprecipitate isolated from a low-spin membrane fraction but not in GLUT4 immunoprecipitate isolated from a high-spin membrane fraction. In the present study, we have taken advantage of this in vitro assay, and we have purified a protein fraction that induces GLUT4 transfer in our assay. This fraction contained a PLD that is known to cleave GPI anchors (7, 15). The cleavage of GPI-linked proteins by GPI-PLD results in production of phosphatidic acid in the large membrane compartment harboring part of the GPI link (see Fig. 9). This may explain our previous finding of an insulin-induced production of phosphatidic acid in the GLUT4 membrane compartment immunoprecipitated from a low-spin membrane fraction (17). By adding PLD inhibitor/activators to the in vitro assay and by isolation of the pure GLUT4 transporter, we provide evidence that the GLUT4-containing vesicles may be GPI linked to a large donor membrane compartment. Upon stimulation, this GPI link may be cleaved, which may release the GLUT4-containing vesicles from binding to membranes.

It should be noted that we used plasma as the source of purifying the GPI-PLD. Unfortunately, we could not immunologically test whether GPI-PLD is found in skeletal muscle, since there are no antibodies against rodent GPI-PLD. Furthermore, because of the contamination of human muscle biopsies with plasma, it is not possible to verify whether the GPI-PLD originates from plasma or the muscle cell itself. However, GPI-PLD mRNA expression is observed in skeletal muscle (20). Furthermore, the production of phosphatidic acid in GLUT4 immunoprecipitates (17), the coisolation of the GPI constituents phosphatidylethanolamine and fatty acids with the pure GLUT4 transporter (Fig. 6), and the effect of suramin and 1,10-phenanthroline on the in vitro GLUT4 transfer carried out with cytosol isolated from rat skeletal muscle (Fig. 3) very strongly indicate the enzymatic involvement of a GPI-PLD in skeletal muscle.

Interestingly, there was no major difference between the potency of using cytosol from basal skeletal muscle compared with insulin-stimulated (1-min) cytosol. This may suggest a constant active GPI-PLD in the prepared cytosol. However, this is probably an artifact caused by homogenization and may not necessarily be a reflection of the in vivo GPI-PLD activity in the intact muscle cell. For instance, the in vivo GPI-PLD activity is related to the concentration of divalent cations (7, 8, 15, 22, 24). In fact, chelating the divalent cations by adding 1,10-phenanthroline in the in vitro GLUT4 assay very potently inhibited the GLUT4 transfer (Fig. 3). Furthermore, GLUT4 transfer was sensitive to the magnesium concentration (17). Although the concentrations of cations in the buffer are made comparable to the intracellular cytosolic concentrations, the true intracellular concentrations may differ because of membrane compartmentalization. Another alternative is that the GPI-PLD is constant activity in vivo in intact cells. If so, the release of GLUT4-containing vesicles may be clamped by another mechanism. We and others have observed docking to and phosphorylation of GLUT4 protein by protein kinase B (PKB)-β in the process of GLUT4 mobilization from membranes (3, 17, 19). This suggests to us that PKB-β (or possibly another protein kinase) may phosphorylate the GLUT4 protein, preparing it for GPI-PLD cleavage. If GLUT4 phosphorylation is a prerequisite for PLD cleavage, the docking of PKB-β could be the regulated step in the GLUT4 mobilization process.

The GPI-PLD also shows sequence similarity with the integrin family (15). This family is known for mechanochemical transduction properties via PI-3 kinase, which may suggest that GPI-PLD could be activated by both insulin and muscle contractions, which are the two physiological stimuli of GLUT4 translocation to the surface membrane of muscle cells. In this regard, it

![Fig. 9. Hypothetical model of a putative GPI link from the large membrane structure to the GLUT4 protein in the vesicle. The link may consist of fatty acids, phosphate, inositol, glucosamine, several mannitol residues, and phosphatidylethanolamine. The PLD cleavage site is shown.](image)
should be noted that the production of phosphatidic acid in the GLUT4 immunoprecipitate was insulin sensitive (17), and furthermore that muscle contractions cause production of phosphatidic acid in rat skeletal muscle in vivo, suggesting activation of a PLD (6). Interestingly, when GPI is added to cells, it mimics many metabolic actions of insulin (25), suggesting that the GPI link has signaling properties of its own (2, 13).

It was observed that GLUT4 had an elution profile where charge was related to a change in molecular mass. It was speculated that this finding could be because of the presence (uncleaved) and absence (cleaved) of a phosphate as part of the GPI link (Fig. 9). Given this, we were also curious to examine the elution profile for GLUT4 isolated from basal and insulin-stimulated skeletal muscle to try to detect a change in molecular mass and the elution profile. Unfortunately, the poor linearity with silver staining and protein content and the strong signal in the Western blot made it difficult to make any measurements regarding small changes in molecular mass/charge and its possible relation to stimulation with insulin. It was also observed that the GLUT4 protein could be separated into two different phases, namely a water phase and a detergent phase. This is at first glance very unexpected for an integral membrane protein. However, it is a very well known observation for GPI-linked proteins (4, 12, 14a), since the link is detergent resistant (11) and contains large and bulky side chains consisting of very water-soluble glucosamine, carbohydrates, and phosphate. Thus the present observations strengthen the concept that GLUT4 protein contains a GPI link. Finally, recent reports suggest that insulin-stimulated GLUT4 translocation may involve lipid rafts (5), which are detergent-resistant membrane areas enriched in GPI-linked proteins (27).

One question is whether all GLUT4 transporters inside the muscle cell are released via this suggested mechanism. We have used an in vitro assay that may or may not represent the whole population of GLUT4. We have previously done marker enzyme analysis on this membrane fraction (17). However, prior membrane homogenization causes cross-contamination of membranes, and enzyme marker analysis on the recovered membrane fraction does not discriminate between the GLUT4-positive membrane and membranes devoid of GLUT4. Thus we are not in a position to answer this question with the techniques used in the present study. Future studies using microscopy of immunolabeled membrane organelles are necessary to answer this question.

A number of consensus polypeptide sequences are known to signal for the attachment of a GPI (31). This attachment is mediated by a transamidase reaction and takes place during synthesis of the polypeptide in the endoplasmic reticulum. By using computer-based prediction analysis, it was suggested that the COOH terminus of GLUT4 (the amino acid 480, SAT sequence) potentially could have a GPI anchor sequence. However, this prediction is based on calculation after the o,o+2 rule and may produce false-positive results (31). The family of GPI-linked proteins is broad (10), and many GPI-linked proteins are localized on the exterior of the cells, where they can be released in the surrounding milieu. Given this, it might seem surprising that the intracellularly located GLUT4 protein should contain a GPI link. However, also ornithine decarboxylase, GPI proteins in granules, and trafficking of GPI are related to the interior of the cell (1, 10, 12). Furthermore, some aminopeptidases are also known to be GPI linked and released by this mechanism (23). Interestingly, one of the constituents in the GLUT4-containing vesicles is insulin-responsive aminopeptidase (IRAP)/aminopeptidase N. This is one of the few proteins found in the GLUT4 immunoprecipitate that fully translocates together with GLUT4 to the surface membrane. It is tempting to speculate that GLUT4 and IRAP/aminopeptidase are sequestered in the same vesicle because they are both released by cleavage of a GPI link. If IRAP and GLUT4 are indeed sequestered in the same translocating vesicle, it might be argued that in vitro GLUT4 transfer (as observed when adding GPI-PLD to the GLUT4 donor membranes in the present study) may be the result of cleavage of a GPI link to the IRAP rather than to GLUT4. However, our finding of a GPI link on the pure GLUT4 protein indicates that the GPI link indeed is on GLUT4.

Taken together, the present study offers several novel findings. First, it offers further characterization and description of an assay strategy for studying ongoing enzymatic reactions in a demanding tissue. Second, a method was developed for purifying large and pure amounts of GLUT4 proteins enabling GLUT4 immobilization and studies of GLUT4 phosphorylation and binding by other proteins. Third, we have shown that GPI-PLD may cause the release of GLUT4-containing vesicles from an enriched GLUT4 fraction. This GLUT4 release could be activated and inhibited with suramin and 1,10-phenanthroline, compounds known to activate and inhibit GPI-PLD activity, respectively. By purifying the GLUT4 transporter, we also provide immunological and biochemical evidence for a GPI link. In conclusion, we suggest that a GPI-PLD may be a novel and important mediator for GLUT4 mobilization in skeletal muscle.

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