Akt-2 Binds to Glut4-containing Vesicles and Phosphorylates Their Component Proteins in Response to Insulin*

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Glut4-containing vesicles immunoadsorbed from primary rat adipocytes possess endogenous protein kinase activity and phosphorylation substrates. Phosphorylation of several vesicle proteins including Glut4 itself is rapidly activated by insulin. Wortmannin blocks the effect of insulin when added to cells in vitro prior to insulin administration. By means of MonoQ chromatography and Western blot analysis, vesicle-associated protein kinase is identified as Akt-2, a lipid-binding protein kinase involved in insulin signaling. Akt-2 is found to be recruited to Glut4-containing vesicles in response to insulin.

The regulation of postprandial blood glucose levels by insulin is achieved mainly by increased glucose transport into skeletal and cardiac muscle and fat (1, 2). These are the only tissues that express a specific isoform of the glucose transporter, Glut4, which mediates the hormonal effect (for recent reviews see Refs. 3–8). It has been shown that in adipocytes under normal conditions, Glut4 is localized in an intracellular microsomal compartment, “Glut4-containing vesicles,” which are translocated to the plasma membrane in response to insulin. Because total glucose uptake into insulin-sensitive tissues is, in general, proportional to the amount of Glut4 molecules at the cell surface, this translocation process is usually considered as the major mechanism of insulin action on glucose transport.

The protein composition of Glut4-containing vesicles is now rather well characterized. Besides Glut4, they include a novel insulin-regulated aminopeptidase (IRAP), the IGFII/Man 6-phosphate1 receptor, the transferrin receptor, and a recently cloned protein, sortilin (reviewed in Refs. 9 and 10; see also Refs. 11 and 12). These proteins, which have extracellular functional domains, represent major constituents of Glut4-containing vesicles as shown by silver and Coomassie staining (13, 14). In addition to these major “cargo” proteins, Glut4-vesicles are enriched with peripheral and integral membrane proteins that are thought to be involved in membrane trafficking and fusion, such as vesicle-associated membrane protein-2 (VAMP2), cellubrevin, secretory carrier-associated membrane proteins (SCAMPs), low molecular mass GTP-binding proteins, phosphatidylinositol kinases, and several others (reviewed in Refs. 9 and 10). Although the biochemical mechanism of translocation of Glut4-containing vesicles to the cell surface is still unknown, there is evidence suggesting that these vesicles represent a subcompartment of the endosomal system in insulin-sensitive cells in which recycling is inhibited under basal conditions, i.e. in the absence of hormone (14). Insulin administration to cells may release this trafficking block by, for example, removal of an endogenous inhibitor from Glut4-containing vesicles, or by disassociating these vesicles from an intracellular anchor, thus leading to their default fusion with the plasma membrane. Indirect support for this hypothesis derives from recent data showing that the introduction of the cytoplasmic portion of several vesicular proteins, such as Glut4 (15) or IRAP (16), causes Glut4 translocation to the plasma membrane, presumably as a result of competing with the endogenous proteins for the putative inhibitor or anchoring protein. After the trafficking block is released, Glut4-vesicles fuse with the plasma membrane, most likely, via a v-SNARE/t-SNARE-mediated process (reviewed in Ref. 8).

A major question in the field that still remains to be resolved is the signal transduction pathway that couples activated insulin receptor with the Glut4-containing compartment (vesicles) and triggers its recruitment to the plasma membrane. At present, we know only about the upstream part of this pathway: insulin receptor, insulin receptor substrates, PI 3-kinase, PDK1, and Akt/PK B (17). The downstream signaling components, proximal to Glut4-vesicles, remain unknown.

We show here that Glut4-containing vesicles immunoadsorbed from rat adipocytes possess a tightly associated protein kinase activity and several phosphorylation substrates. The vesicle-associated protein kinase has been identified as Akt-2 by MonoQ chromatography and Western blot analysis. Phosphorylation of vesicle component proteins as well as artificial substrates by the vesicle-associated protein kinase is rapidly increased by insulin in a wortmannin-sensitive fashion. These data may provide a missing link in the insulin signal transduction pathway by directly coupling Glut4-containing vesicles to the previously established enzymatic cascade.

MATERIALS AND METHODS

Antibodies—In the present study, we used the monoclonal anti-Glut4 antibody IF8 (18), the anti-SCAMPs antibody 3F8 (19), and the anti-Akt-2 sheep antibody (Upstate Biotechnology, Lake Placid, NY).

Isolation and Fractionation of Rat Adipocytes—Adipocytes were isolated from the epidymal fat pads of male Sprague-Dawley rats (200–250 g) by collagenase digestion (20) and transferred to KRP buffer (12.5 mM HEPES, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO4, 1 mM CaCl2, 0.6 mM Na3HPO4, 0.4 mM NaH2PO4, 2.5 mM d-glucose, 2% bovine serum albumin, pH 7.4). Insulin was administered to cells (where indicated) to a final concentration of 2 μM for 5 min, cells were washed three or four times with HES buffer cooled to 14–16 °C (20 mM HEPES, 250 mM sucrose, 1 mM EDTA, 5 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin, 1 μM aprotinin, 1 μM leupeptin, pH 7.4),
homogenized with a Potter-Elvehjem Teflon pestle, and subcellular fractions were prepared as described previously (21). In some experiments, phosphatase inhibitors (a mixture of 25 mM Na$_2$P$_2$O$_7$, 50 mM NaF, 5 mM Na$_3$VO$_4$) were added to homogenization buffer with no significant effect. Isolated membrane fractions were resuspended in PBS, which contained all of the protease inhibitors listed above.

**Immunoadsorption of GLUT4-containing Vesicles—**Protein A-purified 1F8 antibody, as well as nonspecific mouse IgG (Sigma), were each coupled to acrylic beads (Reacti-gel GF 2000, Pierce) at a concentration 0.4 and 0.6 mg of antibody/mL of resin, respectively, according to the manufacturer’s instructions. Before usage, the beads were saturated with 2% bovine serum albumin in PBS for at least 1 h and washed with PBS. The light microsomes (LMs) from rat adipocytes were incubated separately with each of the specific and nonspecific antibody-coupled beads overnight at 4 °C. The beads were washed twice with PBS and twice with protein kinase buffer (10 mM Tris, 10 mM KCl, 100 mM NaCl, 5 mM MgCl$_2$, pH 7.8), and the adsorbed material was used for phosphorylation experiments as described in the following paragraph.

**Phosphorylation of GLUT4-containing Vesicles—**[γ-32P]ATP (50–100 μM, 300–5000 cpm/pmol) and phosphorylation substrates (as specified separately for each experiment) were added to GLUT4-containing vesicles immunoadsorbed on the beads (see the previous paragraph) in protein kinase buffer. To provide an efficient mixing, the volume of the liquid phase exceeded the volume of settled beads 2-fold. This suspension was intensively shaken for 30 min at room temperature, and immunobeads were separated from the liquid phase. Beads were then washed twice from the excess of radioactive ATP with protein kinase buffer and 10 mM Tris, pH 7.4, and eluted with either 1% Triton X-100 in protein kinase buffer or Laemmli sample buffer without 2-mercaptoethanol. In the experiments when exogenous protein substrates were added to protein kinase assay, 50-μl aliquots of the liquid phase were applied on 2 × 2 cm squares of Whatman P81 chromatography paper, washed three times with 75 mM phosphoric acid, and counted in a scintillation counter by Cherenkov. Alternatively, phosphorylated proteins were electrophoresed, and dried gels were stored in a storage phosphor screen cassette and quantitated in a PhosphorImager (Molecular Dynamics).

**Protein Content—**Protein content was determined with a BCA kit (Pierce) according to manufacturer’s instructions.

**RESULTS**

**Glut4-containing Vesicles Contain an Insulin-stimulated Protein Kinase Activity and Phosphorylation Substrates—**Glut4-containing vesicles were immunoadsorbed from intracellular membranes of rat adipose cells treated and not treated with insulin, and [γ-32P]ATP was added directly to the material adsorbed on the beads as described under “Materials and Methods.” Under these conditions, radioactive phosphate is incorporated into several proteins in Glut4-vesicles. Although the pattern of minor phosphorylated proteins varies to some extent in different experiments, we consistently detect phosphorylation of polypeptides with molecular masses 110, 50, 39, and 25 kDa (Fig. 1A). Insulin stimulation of adipocytes for 15 min results in the significant (p < 0.01) increase in the incorporation of radioactive phosphate into these proteins 1.8 ± 0.3, 2.1 ± 0.5, 2.8 ± 0.4, and 3.2 ± 1.5-fold, correspondingly. The electrophoretic mobility of three of the phosphorylated substrates completely matches that of the major constituents of Glut4-vesicles, gp110, or sortilin (11), Glut4, and the high molecular mass isoform of the SCAMP triplet, p59 (19) (Fig. 1A). To further identify these proteins, 1F8-bound material from insulin-stimulated cells was subsequently eluted with PBS containing 1% Triton X-100 and, after that, with Laemmli sample buffer. As we have shown earlier (13), under these conditions, all vesicular proteins with the exception for Glut4 can be recovered in the Triton eluate, whereas Glut4 is resistant to Triton elution and can be removed from immunobeads only with SDS-containing Laemmli sample buffer. Triton eluate from 1F8 beads was used for immunoprecipitation of SCAMPs
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Materials and Methods

We used exogenous substrates to study the substrate specificity of vesicle-associated protein kinase. LMs (0.15 mg of protein) from adipocytes untreated and treated with insulin for 15 min were immunoadsorbed with 50 µl of 1F8-coupled beads and phosphorylated in vitro as described under "Materials and Methods" using the following exogenous substrates: 1, none; 2, synthetic peptide (165 µg) corresponding to phosphorylation site (Asn600-Leu615) on p85 subunit of PI 3-kinase; 3, dephosphorylated MBP (35 µg, Sigma); 4, total histones (86 µg, Sigma); 5, myelin basic protein (86 µg, Sigma). The figure shows the mean values ± S.E. of three independent experiments. Absence of the error signs on some bars indicates that the error is virtually undetectable.

According to the previously published protocol (19), as expected, phosphorylated Glut4 was detected in the SDS eluate from 1F8 beads, whereas phosphorylated SCAMPs were solubilized in 1% Triton X-100 and were immunoprecipitated with the specific antibodies (Fig. 1B). The nature of the low molecular mass phosphorylated protein (p25) remains unknown (see “Discussion”). Several other phosphorylated bands can be noticed on the autoradiogram shown in Fig. 1A, including those with molecular masses over 200 kDa and below 20 kDa. Electrophoretic mobilities of these proteins correspond to that of the known components of Glut4-vesicles: the IGFII/Man 6-phosphate receptor (p230) (23) and VAMP (p18) (24). We are currently trying to identify these proteins.

Along with protein kinase(s), Glut4-containing vesicles may also contain an endogenous phosphatase activity, which may alter the results of the in vitro phosphorylation. To check this possibility, Glut4-vesicles were immunoadsorbed from insulin-treated and untreated cells, phosphorylated in vitro as described above, thoroughly washed of radioactive ATP, and incubated under the same conditions for another hour. This additional incubation in the absence of ATP does not change the total pattern of phosphorylation or specific incorporation of radioactive phosphate into individual proteins (not shown).

In the next experiments, we explored the substrate specificity of the vesicle-associated protein kinase. As is shown in Fig. 2, myelin basic protein (MBP) was phosphorylated to a greater extent than other substrates analyzed. A small amount incorporation of radioactive phosphate into total fraction of histones (Sigma) was also detected. On the other hand, neither casein nor a synthetic peptide corresponding to the phosphorylation site on the regulatory p85 subunit of PI 3-kinase (25) was phosphorylated by a vesicle-associated protein kinase (Fig. 2), although it could be phosphorylated by immunoprecipitated PI 3-kinase.2 This, together with other evidence (see Fig. 5), suggests that PI 3-kinase which, in a recent study, was found to be associated with Glut4-containing vesicles in an insulin-dependent manner (26), does not phosphorylate their component proteins in vitro. It was also found that the vesicle-associated protein kinase is fairly specific for ATP and cannot use GTP, taken at an equal concentration (data not shown).

As an additional control for phosphatase activity, in vitro phosphorylated MBP was incubated with Glut4-containing vesicles immunoisolated from insulin-treated and not treated cells. No dephosphorylation of 32P-labeled MBP was detected under these conditions (not shown).

Because cytosolic proteins can, theoretically, be nonspecifically associated with the immunoadsorbed material and dissociate with an increase in the ionic strength of the washing buffer, it seemed essential to determine how tightly the endogenous protein kinase is associated with Glut4-containing vesicles. Fig. 3 shows that extensive wash of immunoadsorbed Glut4-containing vesicles with a high ionic strength buffer cannot remove endogenous protein kinase activity from this compartment and does not change the pattern of phosphorylated proteins.

As is shown in Figs. 1–3, some increase in the activity of the vesicle-associated protein kinase always takes place after adipocytes are stimulated by insulin for 15 min. This time point was chosen because the effect of insulin on Glut4 recruitment to the plasma membrane reaches the maximum at about this time (for recent study see Ref. 27). However, activation of the vesicle-associated protein kinase may precede vesicle translocation. Thus, we immunoadsorbed Glut4-containing vesicles from adipocytes treated with insulin for 2, 4, 8, and 16 min and determined the level of protein kinase activity in this material. Fig. 4A shows that phosphorylation of the vesicle proteins by the endogenous protein kinase is rapidly increased by insulin and then gradually declines after 2 min of insulin stimulation.

In this and other (Figs. 1, 3, and 5) experiments, however, the effect of insulin on phosphorylation of Glut4-containing vesicles in vitro may and should depend on the phosphorylation status of the substrate proteins in living cells, which is hard if not impossible to measure. In other words, if insulin causes dephosphorylation of Glut4-containing vesicles in vivo, we may see an increase in their phosphorylation in vitro even if the level of the vesicle-associated protein kinase activity stays the same. To distinguish between these two possibilities, we measured the activity of this protein kinase toward an exogenous substrate, MBP (Fig. 4B). Because the pattern of MBP phosphorylation mirrors the incorporation of radioactive phosphate into component proteins of Glut4-vesicles (Fig. 4A) and given the lack of the detectable phosphatase in this preparation, we conclude that protein kinase activity associated with Glut4-vesicles is indeed stimulated by insulin with the maximum at 2 min after insulin administration. Thus, in all following experiments, the effect of insulin on phosphorylation of Glut4-vesicles was measured after 2 min of insulin treatment.

To determine whether vesicle-associated protein kinase may participate in the transduction of the hormonal signal from the cell surface to its final target: Glut4-containing vesicles, we carried out experiments with wortmannin, a specific inhibitor of PI 3-kinase and its downstream signaling. The left panel of Fig. 5 demonstrates that the addition of wortmannin (5 µM) to the in vitro protein kinase assay has no effect on phosphorylation of vesicle proteins by the endogenous protein kinase. This result provides additional evidence that Ser/Thr kinase activity of PI 3-kinase is not responsible for phosphorylation of Glut4-vesicles. In analogous experiments, we have shown that neither PK I (an inhibitor of protein kinase A) nor calphostin C (an inhibitor of protein kinase C) has any effect on the pattern of phosphorylated proteins in Glut4-vesicles (not shown). In contrast, wortmannin in a much lower concentration (100 nM) was able to prevent insulin-stimulated increase in phosphorylation of different proteins in Glut4-vesicles when added to adipose cells in vivo, 20 min prior to insulin administration (Fig. 5, right panel, and Table I).

Identification of the Vesicle-associated Protein Kinase Activity as Akt-2—Data shown in Figs. 4 and 5 suggest that the

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vesicle-associated protein kinase may participate in the insulin signaling downstream of PI 3-kinase. Moreover, in these experiments we have detected a protein with the molecular mass of 60 kDa, the phosphorylation of which is transiently activated by insulin in a wortmannin-sensitive fashion. After 2 min of insulin stimulation, p60 represents one of the major phosphorylated proteins in Glut4-vesicles, whereas after 15 min of insulin treatment its phosphorylation is hardly detectable (compare Figs. 4 and 5 with Figs. 1 and 3). The molecular mass of this protein corresponds well to that of the newly described lipid-binding Ser/Thr protein kinase, Akt. As has recently been shown, Akt is activated by insulin in adipose and muscle cells (28–31), is located downstream of PI 3-kinase (reviewed in Ref. 17), and may mediate the effect of insulin on Glut4 translocation (32–35). Therefore, we decided to check whether Akt is directly present in Glut4-containing vesicles. As is seen in Fig. 6, Glut4-vesicles include visible amounts of Akt-2, an isoform of the enzyme that is predominant in adipocytes (36). These data correspond well to recent results of Calera et al. (37), who demonstrated by immunoadsorption and sucrose gradient centrifugation that Akt-2 may interact with Glut4-containing vesicles in an insulin- and wortmannin-sensitive fashion. Note, that according to Western blot analysis, only a small portion (5–7%) of the total cellular Akt-2 was recovered in the fraction of light microsomes with the major pool of the enzyme being localized in the cytosol. Of this membrane-associated enzyme, 3–4% was found in Glut4-containing vesicles (Fig. 6). However, it is still an open question as to what portion of the LM fraction represents membranes and what portion is accounted for by ribosomes, cytoskeleton, heavy protein complexes, etc., that are also pelleted under conditions used to purify so-called “light microsomes” (38). Thus, Fig. 6 may considerably underrepresent the fraction of the total membrane-associated Akt-2 that is present in Glut4-containing vesicles.

Glut4-containing vesicles from insulin-treated cells contain at least three times more Akt-2 than vesicles from basal cells (Fig. 6). This is consistent with our data about insulin-stimulated increase in phosphorylation of vesicle proteins (Figs. 1–5) and suggests that this phenomenon is likely to be explained by recruiting more active Akt-2 onto Glut4-vesicles.

To estimate to what extent Akt-2 associated with Glut4-containing vesicles can account for the total protein kinase activity present in this compartment, we immunoadsorbed Glut4-containing vesicles on 1F8 beads, solubilized their component proteins with 1% Triton, fractionated this material on a MonoQ column, and determined protein kinase activity in the chromatographic fractions using MBP as a substrate (Fig. 7, top panel). In parallel, we fractionated Triton-solubilized total LMs from adipose cells on the same column under the same experimental conditions and determined the position of Akt-2

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**Fig. 3. Salt resistance of the vesicle-associated protein kinase.** LMs (0.15 mg of protein) from adipocytes untreated (−) and treated with insulin for 15 min (+) were immunoadsorbed with 50 μl of 1F8- or IgG-coupled beads. Immunobeads were washed twice with PBS with final salt concentration corresponding to 0.1, 0.25, and 0.5 M NaCl and, after that, twice with protein kinase buffer (see "Materials and Methods"). Phosphorylation assay was then carried out in the absence (A) or in the presence (B) of 86 μg of myelin basic protein, as described under "Materials and Methods." A shows a representative result of three independent experiments (insulin-treated cells only). The positions of the molecular mass standards are shown on the right. B shows the mean values ± S.E. of three independent experiments. The results of nonspecific binding (not shown) were subtracted from the experimental values.

**Fig. 4. Activation of the vesicle-associated protein kinase by insulin; time course.** Rat adipocytes were treated with insulin for specified periods of time and fractionated by differential centrifugation. LMs (0.15 mg of protein) were immunoadsorbed with 50 μl of 1F8- or IgG-coupled beads and phosphorylated in vitro in the absence (A) or in the presence (B) of 86 μg of myelin basic protein, as described under "Materials and Methods." A shows a representative result of three independent experiments. The positions of the molecular mass standards are shown on the right. B shows the mean values ± S.E. of three independent experiments.
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The effect of wortmannin on insulin-stimulated phosphorylation of Glut4-containing vesicles by an endogenous protein kinase

The table represents quantitation of the data shown in Fig. 5 (right panel). It demonstrates the increase in phosphorylation of the individual proteins of Glut4-containing vesicles (fold stimulation) by an endogenous kinase after insulin administration to adipocytes for 2 min in the absence and in the presence of 100 nM wortmannin. The numbers are the means ± S.E. of four independent experiments.

| Protein     | − Wortmannin | + Wortmannin |
|-------------|--------------|--------------|
| p110        | 3.5 ± 2.1    | 0.9 ± 0.3    |
| p60         | 7.6 ± 0.9    | 1.1 ± 0.2    |
| p50 (Glut4) | 6.2 ± 1.8    | 1.1 ± 0.4    |
| p39 (SCAMP) | 4.8 ± 0.9    | 1.2 ± 0.3    |
| p25         | 5.3 ± 1.5    | 1.3 ± 0.4    |

Discussion

Results presented here demonstrate that after insulin stimulation of native rat adipocytes, Akt-2 is rapidly recruited to Glut4-containing vesicles where it can phosphorylate several component proteins of this compartment including Glut4 itself.

Because the amount of Akt-2 recruited onto Glut4-vesicles is rather low, an important question is the stoichiometry of phosphorylation of Glut4-vesicles by this protein kinase. Unfortunately, such calculations are not very accurate, because it is virtually impossible to estimate the amount of individual proteins in Glut4-vesicles. We know, however, that when we were isolating individual proteins from these vesicles for sequencing, we obtained from about 50 mg of LMs, 23 pmol of IRAP (gp160), 8 pmol of the IGFII/Man 6-phosphate receptor (13), and 8 pmol of sortilin (11). For this work, we take for immunoadsorption

Table 1

The effect of wortmannin on insulin-stimulated phosphorylation of Glut4-containing vesicles by an endogenous protein kinase

Fig. 5. Wortmannin inhibits insulin-dependent activation of the vesicle-associated protein kinase in vivo, but not in vitro. Left panel, LMs (0.15 mg of protein) from adipocytes untreated and treated with insulin for 2 min were immunoadsorbed with 50 μl of 1F8 beads and phosphorylated in vitro in the absence or in the presence of 5 μl wortmannin. Right panel, adipocytes were pre-incubated with 100 nM wortmannin (where indicated) for 20 min before insulin administration for 2 min, and Glut4-vesicles were immunoadsorbed and phosphorylated in vitro as described in the legend to the left panel. The positions of the molecular mass standards are shown on the right. A representative result of four independent experiments is shown. Quantitation of the results shown in the right panel of the figure is presented in Table I.

Fig. 6. Akt-2 is specifically present in Glut4-containing vesicles from rat adipocytes. LMs (0.15 mg of protein) from adipocytes untreated and treated with insulin for 2 min were immunoadsorbed with 50 μl of 1F8 or IgG beads. Eluted material along with original LMs (100 μg) and cytosol (Cyt) (100 μg) was analyzed by Western blot with anti-Akt-2 and anti-Glut4 antibodies. A representative result of three independent experiments is shown.

Fig. 7. Vesicle-associated protein kinase co-fractionates with Akt-2 on a MonoQ column. Top panel, LMs (1.4 mg of protein) from adipocytes treated with insulin for 2 min were immunoadsorbed with 400 μl of 1F8 beads and eluted with 1.6 ml of 1% Triton X-100 in protein kinase buffer. Eluted material was fractionated on a MonoQ column (Amersham Pharmacia Biotech) as described under “Materials and Methods.” Protein kinase activity was measured in aliquots (70 μl) of the gradient fractions with MBP (10 μg) as a substrate. After phosphorylation, samples were electrophoresed, and MBP-incorporated radioactivity was measured in a PhosphorImager. Bottom panel, LMs (1 mg) from adipocytes treated with insulin for 2 min were solubilized in 1% Triton X-100 and fractionated on a MonoQ column in parallel with the material eluted from 1F8 beads. Aliquots (120 μl) of even gradient fractions were analyzed by Western blotting with anti-Akt-2 antibody. Triton-solubilized LMs (40 μg) was loaded on the first lane of the gel as a reference. A representative result of three independent experiments is shown.
on the PhorImager. The positions of the molecular mass standards are shown.

was electrophoresed, and radioactive proteins were analyzed in a Phos-

buffer to the protein kinase assay where indicated. After phosphoryla-

containing fraction 10 (Fig. 8) was added instead of an equal volume of

is shown.

LMs (0.2 mg of protein) from adipocytes untreated and treated with insulin for 2 min were immuno-

to the plasma membrane.

fractionated on a MonoQ column (Amersham Pharmacia Biotech) as described under "Materials and Methods." Fractions were analyzed for the total protein content (upper panel), and aliquots (120 µl) of even fractions were analyzed by Western blotting with anti-Akt-2 antibody (bottom panel). A representative result of five independent experiments is shown.

FIG. 8. Purification of Akt-2 from cytosol on a MonoQ column. Cytosol (5.2 mg) from adipocytes treated with insulin for 2 min was fractionated on a MonoQ column (Amersham Pharmacia Biotech) as described under "Materials and Methods." Fractions were analyzed for the total protein content (upper panel), and aliquots (120 µl) of even fractions were analyzed by Western blotting with anti-Akt-2 antibody (bottom panel). A representative result of five independent experiments is shown.

FIG. 9. Exogenous Akt-2 can phosphorylate component proteins of Glut4-containing vesicles. LMs (0.2 mg of protein) from adipocytes untreated and treated with insulin for 2 min were immuno-

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150 µg of LMs, so we are likely to obtain not more than 0.01–0.1 pmol of the individual proteins. Usually, specific activity of ATP in our assay is 2000 cpm/pm, so if incorporation of $^{32}$P is 1:1, there should be 20–200 cpm in a band. This is about what we detect. It must also be considered that IRAP, the IGFII/Man 6-phosphate receptor, and sortilin are the major proteins in these vesicles, and the amount of SCAMPs or p25 may be lower by an order of magnitude, because we were never even able to see these proteins by silver staining. Lastly, phosphorylation in our assay takes place not in solution but rather on the surface of the vesicles (see below), which theoretically should decrease the efficiency of phosphorylation. So we believe that the specific incorporation of radioactive phosphate into individual component proteins of Glut4-vesicles in vitro is at least 1:1.

Phosphorylation of Glut4 has previously been detected and studied in several laboratories (39–44). In these studies, only one phosphorylation site (Ser$^{488}$) was found on the cytoplasmic C terminus of the transporter molecule (39, 40). However, no insulin-dependent increase in Glut4 phosphorylation was detected in vitro (39, 40). On the other hand, it has been shown that mutation of Ser$^{488}$ as well as the following double leucine motif (Leu$^{489}$-Leu$^{490}$) significantly impairs Glut4 internalization and targeting (reviewed in Refs. 5 and 8). Other evidence indicates that translocation of Glut4 from an intracellular pool to the plasma membrane is accompanied by an unknown modification of the C terminus of the transporter. This causes “unmasking” of this region of the Glut4 molecule as revealed by the increased binding of antibodies (45, 46). It has also been suggested that a regulatory protein may interact with the C terminus of Glut4 in an insulin-dependent manner and thus control its intracellular trafficking (15).

Under the conditions of phosphorylation in the experiments presented here (intact vesicles attached to immunobeads), radioactive phosphate can be incorporated only into cytoplasmic portions of Glut4 and other substrates by a peripherally associated protein kinase. Ser-488 is localized in the cytoplasmic tail of Glut4 and lies in Arg-Xaa-Xaa-Ser-Leu motif, which represents a phosphorylation site of Akt. It seems likely, therefore, that this residue may be a target of Akt associated with Glut4-containing vesicles. It will be interesting to check this hypothesis experimentally and to determine whether phosphorylation of Glut4 may play a role in the unmasking of the C terminus of the transporter and its interaction with a putative regulator.

Identification of other phosphorylation substrates in Glut4-vesicles, such as low molecular mass proteins p25 and p18/21, may also be of great importance for further understanding of insulin signaling. An attractive hypothesis is that p25 represents one of the low molecular mass GTP-binding proteins, and p18/21 may be VAMP and/or cellubrevin. Glut4-vesicles were reported to contain Rab4 (48), which, in addition, may be phosphorylated in response to insulin (49). Although we do not see any significant enrichment of Rab4 in Glut4-containing vesicles, p25 may still represent some other member of the Rab family of similar electrophoretic mobility. As it has recently been demonstrated, VAMP can be phosphorylated in vitro by calcium/calmodulin-dependent protein kinase II (50), although the functional role of this phosphorylation remains unknown.

The potential biological significance of sortilin and SCAMPs

FIG. 10. A proposed insulin signaling pathway in fat cells. Glut4-containing vesicles possess a high basal level of PI 4-kinase activity (51). Insulin stimulation causes rapid targeting of PI 3-kinase to this compartment (26). These two enzymes working together produce phosphatidylinositol phosphates that serve as docking sites for the recruitment and activation of Akt. The latter phosphorylates vesicle proteins, which leads to disassociation of vesicles from an intracellular anchor and their default fusion with the plasma membrane.

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K. V. Kandror and P. F. Pilch, unpublished observations.
phosphorylation is also not known. We are now trying to determine whether regulated secretion of other membrane compartments in different cells is accompanied by phosphorylation of these proteins.

So, what can be a mechanism for the recruitment of Akt-2 onto Glut4-vesicles? As it has been shown previously, the latter have high levels of PI 4-kinase activity (51), and PI 3-kinase is targeted to these vesicles in response to insulin (26). Thus, after insulin stimulation, Glut4-containing vesicles should accumulate phosphatidylinositol 3,4,5-trisphosphates and phosphatidylinositol 3,4-bisphosphates, which serve as additional binding sites for Akt (52–54). This hypothesis is consistent with previous studies demonstrating the importance of membrane recruitment for the activation and function of this enzyme (32, 55, 56). Binding of Akt to Glut4-vesicles may lead to an increase in phosphorylation of their component proteins. Furthermore, it is tempting to propose that this regulatory event may affect interaction of Glut4-vesicles with a putative trafficking inhibitor (see the Introduction) and thus trigger exocytosis (Fig. 10).

The role of Akt in activation of glucose transport in adipose cells has recently been questioned based on the inability of the dominant-negative Akt mutant to inhibit this process in transfected 3T3-L1 cells (57). However, in another set of experiments, kinase-inactive Akt mutant significantly inhibited insulin-dependent translocation of Glut4 in rat adipose cells (35). Moreover, ceramide appears to inhibit Akt kinase activity in parallel with insulin-stimulated Glut4 translocation (58). We believe, therefore, that Akt does have an important role in propagation of the insulin signal in adipose cells, and our results suggest that it may play an important role in the downstream signaling events proximal to Glut4-containing vesicles.

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