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gp160, a tissue-specific marker for insulin-activated glucose transport

(GLUT4/insulin action/adipocytes)

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ABSTRACT **We have isolated and partially sequenced a M_r 160,000 glycoprotein whose rate of cycling to and from the adipocyte cell surface is enhanced by insulin in a manner apparently identical to the effect of insulin on GLUT4 cycling. Based on the protein sequence, we have prepared an anti-peptide antibody against this protein, gp160. The antibody recognizes a M_r 160,000 protein whose subcellular distribution is identical to that of GLUT4. This was determined by three separate criteria: (*i*) Western blotting of fractionated adipocyte membranes from cells exposed to insulin or not, (*ii*) adsorption of vesicles with anti-GLUT4 antibodies followed by Western blotting, and (*iii*) separation of microsomal vesicles by sucrose velocity and density gradients. By all three criteria, GLUT4 and gp160 are completely colocalized in rat fat cells. Moreover, gp160 can be detected by Western blot only in fat and cardiac and skeletal muscles and was absent from all other tissues tested. Thus, gp160 is an additional marker for physiologically important, insulin-sensitive glucose transport. Its further study at the protein and DNA level may reveal information about the mechanistic details of insulin-activated GLUT4 translocation as well as information concerning the tissue-specific expression of GLUT4 and gp160.**

The regulation of postprandial blood glucose levels by insulin is achieved mainly by increased glucose transport exclusively 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 reviews of glucose transporter isoforms and their expression, see refs. 3–5). The mechanism of glucose transport activation by insulin is the hormone-dependent enhancement of the rate of GLUT4 translocation from intracellular storage vesicles to the plasma membrane in such a way that the concentration of the transporter on the cell surface increases 10- to 40-fold, depending on cell type and the method of measurement (6–10). Glucose uptake is increased proportionally to the increment of GLUT4 molecules in the plasma membrane, suggesting that redistribution of transporters is the main, if not the only, mechanism that accounts for this effect.

Transfection experiments have shown that the forced expression of GLUT4 in heterologous cells, which possess insulin receptors and demonstrate certain responses to insulin, is insufficient to produce a major insulin-induced increase in plasma membrane GLUT4 content and activated glucose transport comparable to that observed in normal adipocytes. Cell lines studied in this fashion include 3T3-L1 adipocytes, Chinese hamster ovary (CHO) fibroblasts, HepG2 hepatomas, and the skeletal muscle-like cells, C2C12 (11–14). Although there is a large gap in our understanding of signal transduction between the activated insulin receptor and GLUT4 translocation, these experiments indicate that insulin unresponsive (with regard to GLUT4 translocation) cells must lack some of the important components that are requisite for insulin-dependent recruitment of GLUT4 to the cell surface.

Analysis of the intracellular compartmentalization of GLUT4 in fat and muscle cells in the absence of insulin reveals that the great majority of the transporter (>90%) is found in membrane vesicles, which can be well separated from cellular light microsomes (LM*) by velocity gradient centrifugation (refs. 15 and 16; unpublished results; Fig. 4*A*). It has been postulated that GLUT4 recycles in cells as a constituent of these vesicles in the process of insulin-regulated exocytosis and endocytosis (17–20). We have recently obtained results in our laboratory that are consistent with this hypothesis by detecting three additional proteins in GLUT4-containing vesicles that cycle to and from the cell surface along with GLUT4 in an insulin-dependent fashion (21). GLUT4-containing vesicles thus represent a crucial part of the molecular machinery responsible for compartmentalization and insulin-sensitive translocation of the transporter. However, the protein composition of GLUT4-containing vesicles is mostly unknown. Recently, several proteins have been identified as their components. These include phosphatidylinositol 4-kinase (22), secretory carrier membrane proteins (SCAMPs; ref. 23), also called GTV3 for glucose transporter vesicle-associated triplet proteins (24), vesicle-associated membrane proteins (VAMPs; ref. 25), and some low molecular weight GTP-binding proteins including rab4 (26, 27). None of these proteins are specific for GLUT4-containing vesicles; rather, they are found in a variety of membrane structures from many different cell types. However, besides some components of widespread distribution, which may determine the most general structural features of different microsomes and their fission and fusion, GLUT4-containing vesicles should also have a set of specific proteins responsible for their unique biological functions and regulation.

By means of cell surface biotinylation, we have previously detected three high molecular weight glycoproteins in rat adipocytes that cycle to and from the cell surface in response to insulin in a manner apparently identical to GLUT4 (21). By the relatively imprecise criteria of silver staining, these proteins appear to represent major components of GLUT4-containing vesicles. One of them, gp160, was isolated in sufficient quantity for protein microsequencing and was found to be a protein whose sequences (23 and 17 amino

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Abbreviations: BSA, bovine serum albumin; HRP, horseradish peroxidase; SCAMPs, secretory carrier membrane proteins; GTV3, glucose transporter vesicle-associated triplet proteins; VAMPs, vesicle-associated membrane proteins.

*HM (heavy microsomes) and LM (light microsomes) have been used instead of traditional abbreviations HDM (high density microsomes) and LDM (low density microsomes) because of the virtually identical buoyant density of both types of microsomes (I. Simpson, personal communication; unpublished data).

acids) are absent from gene data bases (21). Here we demonstrate that gp160 has a subcellular distribution and tissue expression identical to GLUT4 itself, and thus it represents an additional marker protein for GLUT4-containing vesicles and insulin-responsive glucose transport.

MATERIALS AND METHODS

Preparation of Specific Anti-gp160 Antibodies. A 13-mer oligopeptide (LQQERFFPSMQPE) corresponding to the known portion of gp160 was synthesized at the Joslin Diabetes Center (Harvard Medical School). The purified 13-mer was coupled to bovine serum albumin (BSA) with 1-ethyl-3-(3-dimethylyaminopropyl)-carbodiimide (Pierce) and was sent to EastAcres Biological Labs (Southborough, MA) for antiserum production. Specific antiserum giving positive Western blots was partially depleted of anti-BSA antibodies by incubation with BSA immobilized on polyacrylamide beads and was used for Western blotting in a 1:200–1:500 dilution.

Adipocyte Fractionation. Adipocytes were isolated from the epididymal fat pads of male Sprague–Dawley rats (150–175 g) by collagenase digestion (28) and transferred to a modified Krebs–Ringer phosphate buffer (12.5 mM Hepes/120 mM NaCl/6 mM KCl/1.2 mM $MgSO_4$/1 mM $CaCl_2$/0.6 mM Na_2HPO_4/0.4 mM NaH_2PO_4/2.5 mM D-glucose/2% bovine serum albumin, pH 7.4) heated to 37°C. Insulin was administered to cells (where indicated) to final concentration 10 nM for 15 min, and then 0.2 M KCN was added to a final concentration of 2 mM for 5 min. Cells were washed three times with HES buffer (20 mM Hepes/250 mM sucrose/1 mM EDTA/5 mM benzamidine/1 mM phenylmethanesulfonyl fluoride/1 mM pepstatin/1 mM aprotinin/1 mM leupeptin, pH 7.4) at 18°C and homogenized with a Potter–Elvehjem Teflon pestle, and subcellular fractions were prepared as described (29). For further fractionation by velocity or density gradient centrifugation, the LM fraction was pelleted on a 50% sucrose cushion (to avoid a resuspension step) and transferred to PBS containing the cocktail of protease inhibitors described above, by passing through a 5-ml Presto column (Pierce).

Isolation of Crude Membrane Fraction from Different Tissues. Fat, heart, kidney, pancreas, lung, spleen, liver, and brain were obtained from one to three male rats, washed three times with 5 ml of cold HES buffer, and homogenized. Homogenates were spun for 10 min at 5000 rpm, and the supernatant was centrifuged at 48,000 rpm for 1.5 hr on a 50% (wt/vol) sucrose cushion in a Ti-70.1 rotor (Beckman). Pellets were collected from the cushion and resuspended in PBS with protease inhibitors. Purified preparations of intracellular microsomes from skeletal muscle, brain, and liver were kind gifts from Lise Coderre and Galini Thoidis (Boston University Medical School). Protein content was determined with a BCA kit (Pierce) according to manufacturer's instructions for all membranes isolated.

Fractionation of Intracellular Microsomes in Sucrose Gradients. The LM fraction from rat adipocytes in PBS was loaded onto a 4.6-ml continuous sucrose gradient (10%–30% sucrose for velocity centrifugation and 10%–50% sucrose for density gradient centrifugation) and centrifuged, respectively, for 50 min and 18 hr in a SW-50.1 rotor at 48,000 rpm. Each gradient was collected into 26 fractions starting from the bottom of the tube.

Immunoadsorption of GLUT4 Vesicles. Protein A-purified 1F8 antibody (30) and nonspecific mouse IgG (Sigma) were each coupled to acrylic beads (Reacti-Gel GF-2000; Pierce) at a concentration 0.8–1.1 mg of antibody per ml of resin according to manufacturer's instructions. Before usage, the beads were saturated with 2% BSA in PBS for at least 1 hr and washed with PBS. The LM from rat adipocytes were incubated separately with each of the specific and nonspecific antibody-coupled beads overnight at 4°C. The beads were washed five times with PBS, and the adsorbed material was eluted with Laemmli sample buffer without 2-mercaptoethanol.

Gel Electrophoresis and Immunoblotting. Proteins were separated in SDS/polyacrylamide (acrylamide from National Diagnostics) gels according to Laemmli (31) and transferred to an Immobilon-P membrane (Millipore) in 25 mM Tris/192 mM glycine. After transfer, the membrane was blocked with 10% nonfat dry milk in PBS for 2 hr at 37°C. GLUT4 and gp160 were visualized with specific antibodies and ^{125}I-labeled protein A or horseradish peroxidase (HRP)-conjugated secondary antibodies and an enhanced chemiluminescence substrate kit (NEN).

RESULTS

A very useful assay for assessing insulin-dependent translocation of glucose transporters (6, 30) and other proteins (24, 25) in fat cells is Western blotting with appropriate antibodies after fractionation according to Simpson *et al.* (29) of insulin-treated and untreated adipocytes. In Fig. 1, 40-μg aliquots of the membrane fractions indicated were electrophoresed, transferred to a poly(vinylidene difluoride) membrane, and blotted with monoclonal anti-GLUT4 antibody 1F8 (Fig. 1*B*) and the enhanced chemiluminescence reagents; then after exposure of the film, the same poly(vinylidene difluoride) membrane was incubated with anti-peptide gp160 antibody (Fig. 1*A*). In control adipocytes incubated without insulin, gp160 is found only in intracellular LM and HM fractions, while the plasma membrane, cytosol, and a combined fraction of mitochondria and nuclei (M/N) do not have detectable amounts of gp160. After administration of insulin to cells, the amount of gp160 in the plasma membrane increases dramatically, whereas the signals in the HM and LM concomitantly decrease. Fig. 1*B* shows the specific GLUT4 staining of the same fractions, and it is apparent that GLUT4 and gp160 have a similar, if not identical, subcellular distribution both in insulin-treated and untreated adipocytes.

The results of Fig. 1 suggested that gp160 and GLUT4 may be colocalized in the same vesicles. Thus, GLUT4-containing vesicles were immunoadsorbed from rat adipocyte LM with 1F8 antibody covalently immobilized on polyacrylamide beads. As a control for this experiment, we used nonspecific mouse IgG immobilized on the same beads at the same concentration. Staining of immunoadsorbed material with

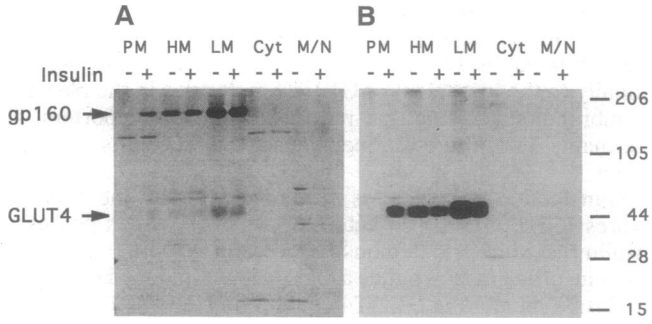

FIG. 1. The subcellular distribution and insulin-responsiveness of gp160 and GLUT4 are identical. Fractions (40 μg of total protein each), obtained as described in *Materials and Methods*, were electrophoresed in 5–15% gradient polyacrylamide gel and analyzed by sequential Western blotting with 1F8 (*B*) and then with anti-gp160 antibodies (*A*). In both cases HRP-conjugated secondary antibodies and a chemiluminescence substrate kit were used for detection. PM, plasma membranes; Cyt, cytosol; M/N, mitochondria and nuclei. The migration of the molecular weight markers ($M_r \times 10^{-3}$) is given at the right.

anti-peptide gp160 antibody demonstrates that this protein is brought down with anti-GLUT4 antibody as a constituent of GLUT4-containing vesicles (Fig. 2). Since equal aliquots of LM from insulin-treated and untreated cells were taken for immunoadsorption, the amount of gp160 (and GLUT4, data not shown) adsorbed on 1F8 beads decreases considerably after insulin administration due to the translocation of GLUT4-containing vesicles from intracellular microsomes to the cell surface and their subsequent fusion with the plasma membrane. Further experiments demonstrated that gp160 is completely eluted from 1F8 beads with 1% Triton X-100 but is resistant to high salt (up to 0.8 M NaCl) washes (results not shown), which indicates that this protein is most probably an integral membrane component of GLUT4-containing vesicles.

To find out what portion of total gp160 is immunoadsorbed with 1F8 beads, we compared the amount of this protein (and GLUT4 itself) in the original LM preparation, immunoadsorbed material, and nonadsorbed supernatant after immunoadsorption (Fig. 3). The recovery of both proteins after elution from 1F8 beads is the same and makes up 70–80% of their total amounts, which is typical for our immunoadsorption procedure. We have concluded from these data that the majority of gp160 is colocalized with GLUT4 in specialized vesicles.

To confirm this result, we separated intracellular LM in velocity and density sucrose gradients. The position of GLUT4-containing vesicles was estimated in fractionated gradients by electrophoresis and Western blotting with specific anti-GLUT4 antibody 1F8. These vesicles have a unique narrow sedimentational and density distribution, which differentiate them from other intracellular microsomes (Fig. 4). Fig. 4*A* demonstrates that GLUT4-containing vesicles sediment faster than the majority of total LM, and the position of gp160 in the gradient is virtually identical to that of GLUT4. The density distribution of the total LM is rather heterogeneous (Fig. 4*B*); however, under these conditions, GLUT4 and gp160 are also localized in particles with an identical buoyant density. Together with coimmunoprecipitation, the complete codistribution of both proteins in sucrose gradients also supports the hypothesis that they are localized in rat adipocytes in the same vesicles.

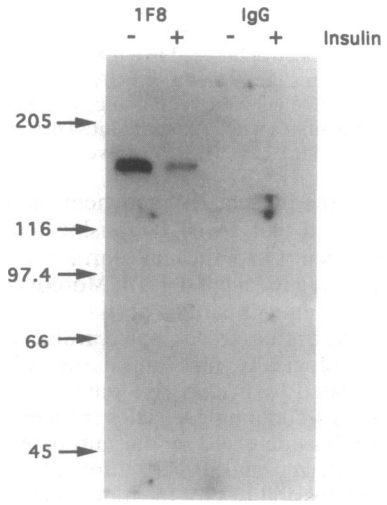

FIG. 2. Anti-GLUT4 antibodies immunoadsorb gp160. LM (0.4 mg each) from insulin-treated and untreated adipocytes were immunoadsorbed with 0.1 ml of settled immunobeads as described in *Materials and Methods*. Elution of protein from the beads was performed with 0.6 ml of Laemmli sample buffer without 2-mercaptoethanol. The eluate (0.1 ml) was analyzed by electrophoresis in a 6.8% gel and Western blotting with ^{125}I-labeled protein A. The migration of the molecular weight markers ($M_r \times 10^{-3}$) is given at the left.

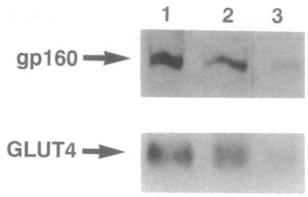

FIG. 3. Anti-GLUT4 antibody immunoadsorbs an identical proportion of GLUT4 and gp160 from microsomal vesicles. The distribution of gp160 and GLUT4 among original LM (lane 1), material immunoadsorbed with 1F8 antibodies (lane 2), and nonadsorbed supernatant after immunoprecipitation (lane 3) is shown. Lane 1 contains 10 μg of LM from basal adipocytes; lanes 2 and 3 contain aliquots of immunoadsorbate and supernatant corresponding to 10 μg of the original LM. The detection system was HRP-conjugated secondary antibodies and a chemiluminescence substrate kit.

It has been shown that the expression of GLUT4 is tissue specific and occurs only in fat and muscle cells (3–5, 30). Fig. 5*A* shows the distribution of gp160 in crude membrane preparations from different tissues. To increase the sensitivity of the analysis, we repeated this experiment with purified fractions of intracellular microsomes isolated from some of the tissues (Fig. 5*B*). Both panels of Fig. 5 demonstrate that gp160 is present in adipocytes, as well as in cardiac and skeletal muscle. In preparations of total membranes from fat and muscle, our antibody picks up a high molecular weight protein, which can be also found in muscle (but not adipocyte) intracellular microsomes. This protein is not present in GLUT4-containing vesicles from both tissues and most probably has no relation to gp160. Membrane preparations from the other tissues taken at the same concentration do not show any presence of gp160 by Western blot analysis with our antibodies. Thus, the expression patterns of GLUT4 and gp160 are identical and are limited to insulin-sensitive tissues, which gives additional evidence to the possible functional importance of gp160 in insulin-sensitive glucose transport.

DISCUSSION

It is now widely accepted that the functioning and regulation of the glucose transporting machinery in insulin-sensitive cells may be described in terms of recycling of GLUT4-containing vesicles between an intracellular pool and the cell surface. Along with this, trafficking of GLUT4-containing vesicles may be considered as an example of a more general and fundamental process of vesicle-mediated molecular transport. An understanding of the molecular mechanism of vesicle trafficking will require identification of structural and regulatory proteins involved in this process. GLUT4-containing vesicles represent a very good model for this study, since they are homogeneous in size (15) and have a very narrow sedimentational and density distribution (Fig. 4) and an apparently constant protein composition. These vesicles carry a unique tag, GLUT4, which makes possible their isolation by immunoadsorption and the analysis of their distribution and fate in living cells.

The proteins previously identified as components of GLUT4-containing vesicles—namely, phosphatidylinositol 4-kinase (22), GTV3/SCAMPs (23, 24), VAMPs (25), and some low molecular weight GTP-binding proteins (26, 27)—did not give any clues as to insulin-specific regulation, since all these proteins have a broad tissue distribution unlike GLUT4 itself. In addition, these proteins (with possible exception for GTV3/SCAMPs) represent only minor components of GLUT4-containing vesicles hardly detectable on silver-stained gels. However, the presence of these proteins in GLUT4 vesicles supports conclusions that there are com-

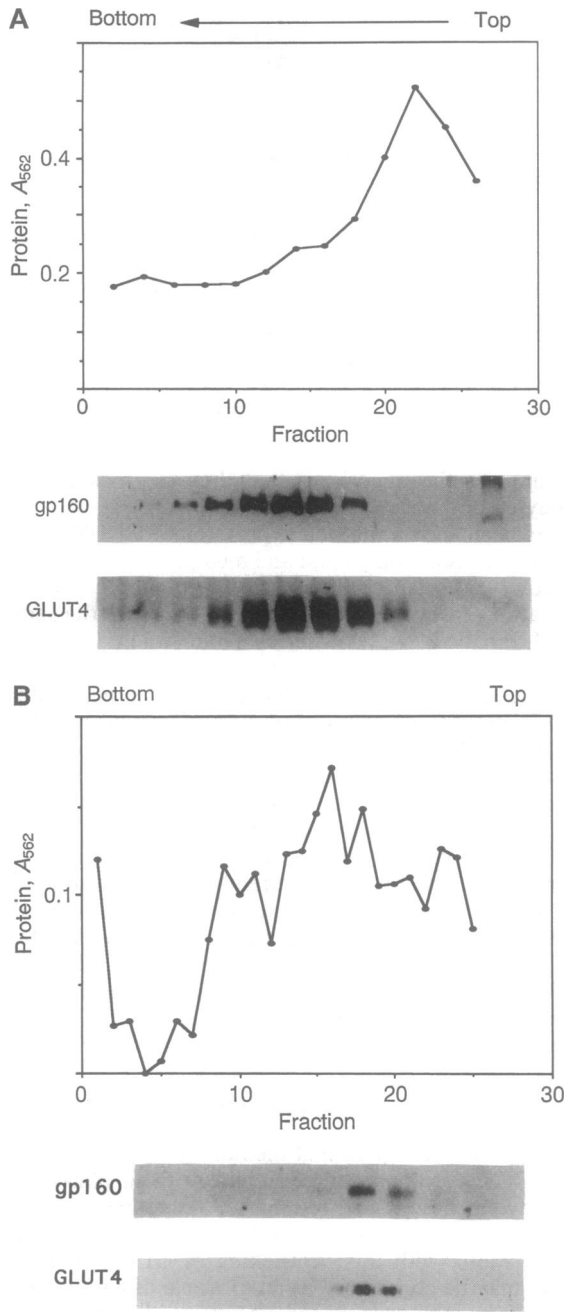

FIG. 4. GLUT4 and gp160 have an identical distribution in velocity (*A*) and density (*B*) sucrose gradients. LM (1.2 mg) from basal adipocytes were loaded on velocity gradient, and 0.11 mg of LM was loaded on a density gradient. The horizontal arrow in *A* shows the direction of sedimentation. After centrifugation as described in *Materials and Methods*, odd-numbered fractions were analyzed by Western blot, and GLUT4 and gp160 were visualized by chemiluminescence.

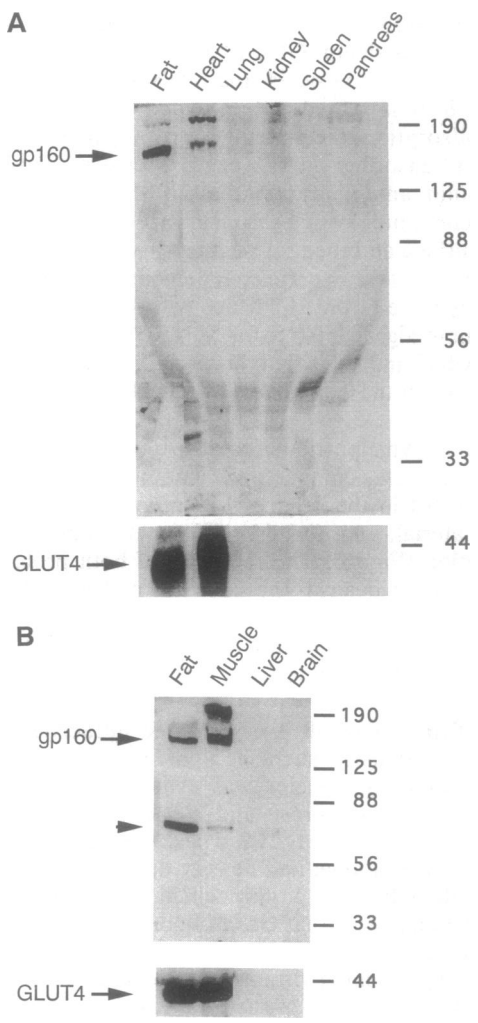

FIG. 5. The expression of gp160 is specific to fat and muscle tissue. (*A*) Total postnuclear membranes (80 μg each) from fat, heart, lung, kidney, spleen, and pancreas were electrophoresed in parallel on two 7.5% polyacrylamide gels; they were blotted and stained with anti-gp160 serum (*Upper*) and 1F8 antibody (*Bottom*). (*B*) Purified intracellular microsomes (10 μg each) from fat, skeletal muscle, brain, and liver were electrophoresed in a 6.8% polyacrylamide gel. The arrowhead indicates the position of BSA. In both cases, gp160 and GLUT4 were detected with HRP-conjugated secondary antibodies and a chemiluminescence substrate kit. The migration of the molecular weight markers ($M_r \times 10^{-3}$) is given at the right.

mon mechanisms in vesicle-mediated secretion, regardless of cell type or physiological processes (32).

To our knowledge, glycoprotein gp160 is the first protein that has been isolated directly from immunopurified GLUT4-containing vesicles. Our rough estimation of the intensity of protein silver staining in polyacrylamide gels and Coomassie staining on poly(vinylidene difluoride) membranes indicates that gp160 is one of the major components of these vesicles, whose amount is roughly similar to that of GLUT4 (21). All data obtained so far (Figs. 1–4) support the hypothesis that in fat cells gp160 is specific to GLUT4-containing vesicles. Our previous experiments on biotinylation of cell surface proteins (21) and the present immunochemical data (Figs. 1 and 2) demonstrated that gp160 is translocated from intracellular compartment(s) to the cell surface in an insulin-dependent fashion identical to GLUT4. Moreover, gp160 and GLUT4 have the identical pattern of tissue-specific expression, which is restricted to fat and cardiac and skeletal muscle, the physiologically important tissues where glucose transport is regulated by insulin. We have recently demonstrated that GLUT4-containing vesicles from muscle protein also contain gp160 as detected by our anti-peptide antibody (K.V.K., L. Coderre, and P.F.P., unpublished results). There are several possibilities for the functional role of gp160. It may be involved in the formation of the GLUT4 vesicles and/or the targeting of GLUT4 to this vesicle. Alternatively, its insulin-dependent recruitment to the cell surface may be an additional requirement for the overall cellular response to insulin and may serve some, as yet unknown, physiological function.

Note. While this manuscript was under review, a paper appeared describing the purification from fat cell microsomes of a glycoprotein

of M_r 165,000 (33). They report a sequence of peptide iii from this protein as XXLINNFELAGL, whereas we sequenced the peptide DRANLINNFELA. Thus, it is likely that the protein we report here and that described by Mastick *et al.* (33) are identical.

Note Added in Proof. A recent computer search revealed some homology between all sequenced peptides of gp160 (ref. 33; this paper) and aminopeptidase N. We have verified that gp160 indeed has aminopeptidase activity *in vitro*.

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