A Synthetic Peptide Corresponding to the GLUT4 C-terminal Cytoplasmic Domain Causes Insulin-like Glucose Transport Stimulation and GLUT4 Recruitment in Rat Adipocytes*

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Glucose uptake by animal cells is catalyzed by a family of integral membrane proteins (the facilitative glucose transporters or GLUTs) and further subjected to tissue-specific hormonal and metabolic regulation (1). By far the most important and best known GLUT regulation is that of GLUT4 by insulin in muscle and fat cells (2, 3). Rat epididymal adipocytes represent the best studied cell system for this GLUT4 regulation. They express GLUT4 as the major GLUT isoform, with a small amount (less than 10%) of GLUT1. Very little of the GLUT4 is constitutively (in the absence of insulin) functional in these cells; more than 95% of GLUT4 is sequestered in an intracellular pool and moves to the plasma membrane in response to insulin (4, 5). This is in contrast to the constitutive distribution of GLUT1; as much as 40% of GLUT1 is already at the plasma membrane, and insulin changes this distribution relatively less (2). It should be noted here that the constitutive sequestration specific to GLUT4 is a necessary prerequisite to its massive insulin-induced recruitment to the plasma membrane and may be considered as part of GLUT4 regulation by insulin.

The molecular mechanism by which GLUT4 in adipocytes is constitutively sequestered in intracellular membranes is unknown and currently is the subject of an intensive investigation (for review see Refs. 3, 6, and 7). All members of the GLUT family have a large (amounting to approximately 30% of the protein mass) cytoplasmic domain that shows a significant amino acid sequence divergence (1, 8), suggesting the importance of this domain in tissue-specific GLUT regulations. This domain is made up of three segments or subdomains, namely the N terminus, the C terminus, and a large central loop between putative transmembrane helices 6 and 7. A series of recent transfection studies using GLUT1-GLUT4 chimeras (9–14) have suggested that the C terminus primarily, but also the N terminus and central loop to some extent, determine constitutive GLUT4 targeting to intracellular membranes. Most of these transfection studies, however, were carried out using cells such as Chinese hamster ovary (9, 13), COS-7 (10, 13), NIH 3T3 (12, 14), PC12 (14), and Xenopus oocyte (11), where glucose transport is not physiologically regulated by insulin. More recently, similar transfection studies have been carried out using insulin-sensitive cultured cells including 3T3-L1 adipocytes (15, 16) and L6 myoblasts (17) and have shown that the GLUT4 C-terminal amino acid sequence of the cytoplasmic domain confers the isoform-specific subcellular targeting of GLUT1 and GLUT4 chimeras.

In the present study, we studied the possible role of the C-terminal cytoplasmic domain of GLUT4 and GLUT1 in the isoform-specific constitutive targeting in rat epididymal adipocytes, a classical insulin target cell. We introduced the synthetic peptides corresponding to the GLUT4 and GLUT1 C-terminal sequences (GLUT4C and GLUT1C, respectively) into adipocytes by polyethylene glycol-induced cell-cell fusion using peptide-preloaded rabbit erythrocyte ghosts and studied the effects of these peptides on glucose transport and native GLUT4 and GLUT1 targeting in host cells. Our fusion protocol by itself did not disturb basal and insulin-stimulated glucose transport and GLUT4 or GLUT1 targeting in host cells. We show that GLUT4C peptide introduced in excess in basal adi-
Adipocytes produces a large, insulin-like recruitment of GLUT4, but not GLUT1, with an equally large stimulation of 3OMG\textsuperscript{1} exchange flux in host cells. The GLUT4C incorporation, however, did not increase the insulin-stimulated GLUT4 recruitment and 3OMG flux any further. We also show that the effect is specific to GLUT4C; GLUT1C peptide incorporation did not cause any effect on GLUT1 and GLUT4 targeting or 3OMG uptake. These findings not only provide, for the first time, an evidence for the importance of the GLUT4 C-terminal cytoplasmic domain in constitutive GLUT4 targeting in rat adipocytes but also predict the presence of a protein in adipocytes that interacts with GLUT4 at its C-terminal cytoplasmic domain and mediates either GLUT4 endocytosis at the plasma membrane or GLUT4 retention in its intracellular storage compartment.

**EXPERIMENTAL PROCEDURES**

**Materials**—Collagenase (Type I) was obtained from Worthington (Freehold, NJ). Insulin (porcine crystalline) and 3OMG were from Sigma. 3-O-Methyl D-glucose was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA). Silicone oil (specific gravity, 0.963; viscosity, 50 centistokes) was purchased from Thomas Scientific (Philadelphia, PA). Protein A-horseradish peroxidase-labeled anti-rabbit IgG was from Zymed Laboratories (San Francisco, CA). All other reagents were from sources stated below and were of reagent grade.

**Peptide Synthesis**—Peptides corresponding to the C-terminal sequences of GLUT4 (18), RVPETGRTFQISAAHFRPSLEQEVK-PFSTELEYLPDEND, and GLUT1 (18), KYPETKGRDFDEIASGFRQQGASSEQDTKEPELEFHPGLADSQV, designated as GLUT4C and GLUT1C, respectively, were synthesized by solid phase method on a Preceptive Biosystems automatic synthesizer using Fmoc strategy. Purities of the peptides were 98% or better.

**Preparation of Peptide-loaded Rabbit Erythrocyte Ghosts**—Rabbit erythrocyte ghosts were prepared by following the procedure described for the preparation of human erythrocyte ghosts (19). Rabbit blood was collected from the ear vein using sodium heparin as an anticoagulant, centrifuged at 3,000 $\times$ g for 5 min to remove the buffy coat, and washed twice with buffer (155 mM NaCl, 10 mM Tris-HCl, pH 7.4). Essentially hemoglobin-free leaky ghosts were obtained by three cycles of hemolysis and washing, each in 1/15 isotonic sodium phosphate buffer (110 mM) for 20 min at room temperature and packed by centrifugation (10,000 $\times$ g for 5 min at 4 °C) to approximately 70% (v/v) ghosts. Leaky ghosts were then loaded with peptides and resealed using a 10-fold dilution of polyethylene glycol; PM, plasma membrane; LDM, low density membrane.

**Preparation of Rat Adipocytes and Fusion with Ghosts**—Adipocytes were isolated from epididymal fat pads of male Sprague-Dawley rats weighing 180–220 g sacrificed by cervical dislocation as described (20). Incorporation of peptides into intact adipocytes was achieved by fusing peptide-loaded resealed rabbit erythrocyte ghosts with intact adipocytes using polyethylene glycol (PEG 8,000; molecular weight, 1000–8000) (21). Typically, adipocytes were washed twice with KRH buffer without bovine serum albumin. 1 ml of adipocytes (40–45% cytocrit) were mixed with a specified amount (ranging from 0.4 to 1.6 ml) of resealed ghosts (70–75% cytocrit) preloaded with peptides in KRH buffer containing 60% PEG 8,000 suspension was added from above and mixed gently for 3 min. The final concentration of PEG 8,000 was 10% after mixing. The mixture was washed using 10 volumes of KRH buffer containing 1% bovine serum albumin and 2 mM Na-glucose, and after gentle mixing, adipocytes were recovered by flotation. This washing procedure was repeated twice more. Adipocytes were stabilized at 37 °C for 60 min prior to functional studies. When specified, adipocytes were treated with insulin for the last 20 min of this stabilization period. Peptide incorporation was adjusted by varying the amounts of pre-loaded ghosts used for fusion with a fixed amount of adipocytes and quantitated by immunoblots.

**Subcellular Fractionation of Adipocytes and Immunoblots**—Plasma membrane (PM) and low density microsomal (LDM) fractions of adipocytes as well as adipocyte cytosol were prepared by subcellular fractionation following procedures previously described (22). The procedure for immunoblots has been also described elsewhere (23). Rabbit anti-GLUT4 and anti-GLUT1 sera were raised against the synthetic peptides corresponding to the C-terminal amino acid sequences, KFSTELEYLPDEND and TPTEELFHPGLADSQV, respectively, and used with protein A-horseradish peroxidase-labeled anti-rabbit IgG as the secondary antibody. Immunoblot intensities were quantitated by densitometry using an analytical scanning system (Molecular Dynamics, Sunnyvale, CA). Incorporation of GLUT4C and GLUT1C peptides into adipocytes after fusion were also quantitated by immunoblotting cytosol using the same antisera mentioned above and expressed in mol based on calibration curves constructed from immunoblots of varying amounts of GLUT4C or GLUT1C peptide. Protein concentrations were determined by the method of Bradford (24) using bovine serum albumin as a standard.

**3OMG Flux Assay**—Equilibrium exchange influx of 3OMG was measured by the oil filtration method (25) using \(^{3}C\)3OMG as a tracer as described previously (22). Adipocytes prior to and after peptide incorporation were first equilibrated with 5 mM 3OMG at 37 °C for 30 min. This chemical equilibration time also allowed cell stabilization and overlapped with the 20 min of insulin (10 $^{-7}$ M) treatment where specified. A 30–60-s tracer equilibration time course was constructed by measuring tracer uptake at six time points and analyzed to calculate $\bar{v}_{T}$, the time required for one-half of complete tracer equilibration, as described (22).

**RESULTS**

We introduced synthetic peptides corresponding to the GLUT4 and GLUT1 C-terminal sequences into rat adipocytes via PEG-induced cell-cell fusion using peptide-loaded rat erythrocyte ghosts as described under “Experimental Procedures.” The fusion procedure used here is similar to that that we previously used for the reconstitution of purified human erythrocyte GLUT1 into adipocytes (21) except that peptide-loaded rabbit erythrocyte ghosts were used in lieu of liposomes. The use of liposomes (or proteoliposomes, whose diameters are 0.5 μm or less) with a large surface area to volume ratio is quite effective in reconstituting integral membrane proteins into host cell plasma membranes but is not suitable for introducing a large amount of a soluble protein into host cell cytosol. The use of erythrocyte ghosts, whose volume to surface area ratio is 10 times as large as that of liposomes, allows one to introduce a large quantity of soluble peptides or proteins upon fusion with only a minimal increase in host cell plasma membrane.

Although the mean diameter of rat adipocytes is almost 10 times as large as that of rabbit erythrocyte ghosts, more than 90% of the adipocyte intracellular space is filled with fat (25).\textsuperscript{2} Therefore the ratio of plasma membrane to the intracellular aqueous space for adipocytes and ghosts are roughly equal. Fusion of every peptide-loaded ghost with an adipocyte would therefore result in injection of a volume of ghost content equal to roughly 1% of adipocyte cytosolic aqueous space accompanied by an incorporation of erythrocyte membrane equivalent to 1% of host cell plasma membrane area. For each fusion experiment, the amounts of incorporated GLUT1C peptides and native GLUT4/1 were assessed separately by immunoblots using particle-free cytosolic fractions and cytosol-free total membrane fractions, respectively, of host cells. As much as 1.1 nmol of GLUT4C or GLUT1C peptide and approximately 20 pmol of native GLUT4 were detected in 5 $\times$ 10\textsuperscript{6} adipocytes (approximately 1 ml of 40–45% packed adipocytes) after fusion by our protocol (not illustrated). This would correspond to an approx-

\textsuperscript{1}The abbreviations used are: 3OMG, 3-O-methyl D-glucose; PEG, polyethylene glycol; PM, plasma membrane; LDM, low density microsome.

\textsuperscript{2}C. Y. Jung, unpublished data.
FIG. 1. Immunoblots of GLUT4 and GLUT1 in PM and LDM isolated from adipocytes after GLUT4C or GLUT1C peptide incorporation and with or without insulin treatment. Peptide-incorporated adipocytes were prepared by fusion (see "Experimental Procedures"). 1.6 ml of packed ghosts preloaded with (+) or without (−) peptides (approximately 200 μM) were used for fusion per ml of packed adipocytes. Adipocytes were then incubated with KRH buffer in the absence (−) or in the presence (+) of 10−7 M insulin for 20 min. PM and LDM were prepared and subjected to immunoblot analysis by applying 20 μg of protein in each lane and developing the blots with GLUT4 and GLUT1 antipeptide antisera (as indicated by GLUT4 and GLUT1 at left margin). Intense immunoreactive bands were seen at a position slightly lower than the 53-kDa molecular marker (indicated by the arrow at right) corresponding to intact GLUT4 or GLUT1 protein whereas no immunoreactive band was detectable at the position corresponding to GLUT4C or GLUT1C peptide. Upper panel, data with GLUT4C peptide incorporation; lower panel, data with GLUT1C peptide incorporation.

FIG. 2. Immunoblots of GLUT4 and GLUT1 in PM and LDM isolated from adipocytes incorporated with increasing amounts of GLUT4C and GLUT1C peptides. Experiments were similar to those shown in Fig. 1 except that 0.4 (C1), 0.8 (C2), or 1.6 (C3) ml of peptide-loaded packed ghosts (approximately 200 μM peptide) were used per ml of packed adipocytes in the fusion protocol. Data from adipocytes fused with ghosts loaded with buffer but no peptide are also shown (GLUT4C or GLUT1C as shown at the bottom of each panel). Upper panel, data with GLUT4C peptide incorporation. The peptide amounts in cell lysates were 0.36 (C1), 0.61 (C2), and 1.06 (C3) nmol/ml of 44% packed adipocytes. Lower panel, data with GLUT1C peptide incorporation. The peptide amounts found in cell lysates were 0.36 (C1), 0.62 (C2), and 1.07 (C3) nmol/ml of 43% packed adipocytes. The lower panel also includes data to illustrate that the adipocytes used here respond to insulin significantly by increasing PM GLUT4 level by approximately 3-fold.

FIG. 3. Quantitative analysis of the effects of GLUT4C and GLUT1C peptide incorporation on adipocyte GLUT4 and GLUT1 subcellular distribution in basal and insulin-stimulated host adipocytes. Data are the mean with S.E. of three independent sets of experiments; each was similar to those illustrated in Figs. 1 and 2. 1.6 ml of 70% packed ghosts preloaded with either buffer only or 200 μM GLUT4C or GLUT1C peptide was used for each ml of 40–45% packed adipocytes for fusion. Data are from adipocytes fused with ghosts loaded with no peptide (Bas) or ghosts loaded with GLUT4C (4C) or GLUT1C (1C) peptide and incubated without (Bas) or with insulin (100 nm) for 20 min (Ins). For PM (solid bars) and LDM (shaded bars), Immunoblot intensities were quantitated by densitometry and expressed relative to those of the adipocytes fused with ghosts loaded with no peptide (Bas).

If the C-terminal cytoplasmic domain of GLUT4 plays a critical role in constitutive GLUT4 targeting in adipocytes as recent transfection experiments suggest (Refs. 9–17; also see Introduction), GLUT4C peptide introduced into adipocytes in excess may affect the native GLUT4 distribution between the plasma membrane and the intracellular pool in host cells. That this is indeed the case illustrated in Figs. 1–3, where GLUT4C peptide introduced in approximately 45-fold molar excess in adipocyte cytosol caused a more than 3-fold increase in the plasma membrane level of GLUT4 in host cells, with a concomitant reduction in intracellular GLUT4 content. In these experiments, the subcellular distribution of GLUT4 and GLUT1 were measured by immunoblotting essentially cytosol-free PM and LDM. GLUT1 distribution was not affected significantly in these experiments (Figs. 1–4), indicating that GLUT4C peptide selectively affects GLUT4 targeting. The effect was dose-dependent, increasing almost linearly with increasing amounts of GLUT4C peptide incorporation (Fig. 4). GLUT1C peptide, when similarly introduced in adipocytes, on the other hand, showed no effect or a negligible effect on either constitutive or insulin-responsive subcellular distribution of GLUT1 (Figs. 1–3) and GLUT4 (Figs. 1–4) in host cells, demonstrating that the effect is specific to GLUT4C peptide.

3 J. S. Hah and C. Y. Jung, unpublished data.
shown.

Lines that GLUT4C peptide of approximately 13-fold molar excess to native of 0.096 protocol in the absence of peptides (Figs. 1–3). These control intensities for PM GLUT4 were expressed relative to those measured for adipocytes fused with ghosts containing no peptide (on ordinate). Each data point represents a single determination. Data for GLUT4C were subjected to the fusion protocol in the absence of peptides. Glut1C (data not illustrated). Thus, adipocytes fully retained their ability to respond to insulin after fusion, although basal flux was without effect. These findings strongly suggest that the effect is not an experimental artifact because it is specific to the GLUT4C peptide effect is dose-dependent with respect to the concentration of GLUT4C peptide in cytosol, increasing the plasma membrane GLUT4 level by more than 4-fold. The effect causes a large redistribution of native GLUT4 from the intracellular pool to the plasma membrane in host cells. This GLUT4C peptide effect is dose-dependent with respect to the concentration of GLUT4C peptide in cytosol, increasing the plasma membrane GLUT4 level by more than 4-fold.

FIG. 4. Relative changes in PM GLUT4 level as a function of GLUT4C or GLUT1C peptide content in adipocytes after incorporation. An increasing amount of peptide was incorporated by fusing an increasing amount (from 0.4 to 1.6 ml, approximately 70% packed) of peptide-loaded ghosts with each ml of 40–45% packed adipocytes as in experiments illustrated in Fig. 2. Peptide incorporated into adipocytes was measured for each fusion experiment by semiquantitative immunoblotting analysis and expressed in nmol/ml 40–43% packed adipocytes (on abscissa) as detailed under “Experimental Procedures.” Blot intensities for PM GLUT4 were expressed relative to those measured for adipocytes fused with ghosts containing no peptide (on ordinate). Each data point represents a single determination. Data for GLUT4C (solid circles) and GLUT1C (solid squares) peptide incorporation are shown. Lines were drawn by eye. It is readily calculated from these data that GLUT4C peptide of approximately 15-fold molar excess to native GLUT4 is required to increase the steady state PM GLUT4 by 2-fold (see text).

Also run in parallel with these experiments are control experiments using adipocytes that were subjected to the fusion protocol in the absence of peptides (Figs. 1–3). These control adipocytes showed a constitutive PM to LDM distribution ratio of 0.096 ± 0.015 for GLUT4 and 0.91 ± 0.12 for GLUT1 (average ± S.D.; n = 3) (data not illustrated) and responded to insulin (10−7 M, 30 min at 37 °C) by showing an increase in PM GLUT4 level by 4–5-fold with a concomitant reduction in LDM GLUT4 level (Figs. 1 and 3). These results demonstrate that the fusion protocol per se did not greatly affect the constitutive and insulin-responsive targeting patterns of both isoforms in host adipocytes.

One advantage of the use of the fusion protocol over chemical and electrical permeabilization protocols for peptide incorporation into cells is that the host cells retain their plasma membrane diffusion barrier after fusion allowing one to study transporter function directly after peptide incorporation. The time courses of 3OMG tracer equilibrium exchange measured on adipocytes after peptide incorporation (Fig. 5) demonstrated that GLUT4C peptide also caused a dose-dependent stimulation of glucose transport in host cells. GLUT1C peptide similarly introduced into adipocytes did not affect 3OMG exchange flux in host cells (not illustrated). Also illustrated (Fig. 5) are the time courses of 3OMG exchange by those adipocytes that were subjected to the fusion protocol in the absence of peptides. These adipocytes showed a half-equilibration time (t1/2) for 3OMG exchange of 46.6 s in the basal state and 9.7 s in the presence of insulin (10−7 M, 30 min). Adipocytes prior to the fusion protocol, on the other hand, showed t1/2 values for 3OMG equilibration of 78 and 8 s, basal and after insulin, respectively (data not illustrated). Thus, adipocytes fully retained their ability to respond to insulin after fusion, although basal flux was significantly increased, most likely due to the additional mechanical agitation inherent in this protocol.

DISCUSSION

We have demonstrated in the present study that when the synthetic peptide with the GLUT4 C-terminal 43-amino acid sequence is introduced into rat epididymal adipocyte cytosol, it causes a large redistribution of native GLUT4 from the intracellular pool to the plasma membrane in host cells. This GLUT4C peptide effect is dose-dependent with respect to the concentration of GLUT4C peptide in cytosol, increasing the plasma membrane GLUT4 level by more than 4-fold. The effect is selective, affecting only GLUT4 distribution and not GLUT1 distribution. Furthermore, the GLUT4 redistribution accompanies an equally large and dose-dependent stimulation of glucose transport function as assessed by 3OMG exchange. The effect is not an experimental artifact because it is specific to the GLUT4 sequence; a similar incorporation of the peptide corresponding to the GLUT1 C-terminal 42-amino acid sequence was without effect. These findings strongly suggest that the GLUT4 cytoplasmic domain is indeed important in constitutive GLUT4 targeting in rat adipocytes, a classical insulin target cell.

GLUT4 in rat adipocytes is known to recycle constantly between the plasma membrane and an intracellular pool with two characteristic first order rate constants, one for endocytosis and the other for exocytosis (23). Thus changes in either or both of these processes can cause changes in the steady state
GLUT4 distribution between these two pools. Insulin was shown to recruit GLUT4 to the plasma membrane by reducing the endocytosis as well as enhancing the exocytosis (23), whereas okadaic acid, which also induces GLUT4 recruitment, does so by enhancing the exocytosis only (26). Such a kinetic characterization is not available at this time for the GLUT4C peptide-induced GLUT4 redistribution described here, although preliminary data indicate (not illustrated) that the peptide effects develop rather quickly, apparently completing within 30 min of exposure to peptide. It is thus possible that the endocytosis, the exocytosis, or both are affected by peptide incorporation.

It is not known at the molecular level how the GLUT4C peptide in adipocyte cytosol induces native GLUT4 redistribution from the storage pool to the plasma membrane. Our findings nevertheless offer useful insights into understanding how GLUT4 targeting is regulated at the molecular level in this classical insulin target cell. A straightforward interpretation of the findings is that there is a protein or proteins that participate in or otherwise affect GLUT4 endocytosis, the exocytosis, or both. Further characterization of this protein or proteins postulated above is yet to be determined. The identification, cloning, and characterization of this regulatory protein will be an important first step toward understanding how insulin regulates glucose transport in muscle and adipose tissue.

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