Temporal Separation of Insulin-stimulated GLUT4/IRAP Vesicle Plasma Membrane Docking and Fusion in 3T3L1 Adipocytes

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Examination of the time and temperature dependence of insulin-stimulated GLUT4/IRAP-containing vesicle trafficking demonstrated an approximate 7-fold increase in the half-time for plasma membrane translocation at 23 °C (t\(\frac{1}{2}\) = −30 min) compared with 37 °C (t\(\frac{1}{2}\) = −4 min) without a significant change in the extent of either GLUT4 or IRAP translocation. Localization of the endogenous GLUT4 and expressed GLUT4-enhanced green fluorescent protein fusion protein in intact 3T3L1 adipocytes demonstrated that at 23 °C there was a time-dependent accumulation of discrete GLUT4-containing vesicles adjacent to the inner face of the cell surface membrane but that was not contiguous and/or physically incorporated into the plasma membrane. Together, these data demonstrate that the temperature-dependent decrease in the rate of GLUT4 and IRAP translocation results from a reduction in GLUT4/IRAP-containing vesicle fusion and not trafficking or docking to the plasma membrane.

One of the major downstream biological responses following insulin stimulation is a marked increase in the rate of glucose transport in muscle and adipose tissue. This primarily results from an insulin-dependent translocation of the insulin-responsive glucose transporter isoform (GLUT4) from an intracellular vesicular storage sites to the plasma membrane (1, 2). In addition, these GLUT4-containing vesicular compartments are also enriched for another cargo protein termed IRAP (insulin-responsive amino peptidase) (3–7). Although the molecular mechanism and signaling cascade(s) regulating the intracellular trafficking of GLUT4/IRAP-containing vesicles have not been completely elucidated, these events have several features in common with the regulated exocytotic pathway for neurotransmitter release from synaptic vesicles (8, 9).

In this process, protein complexes in the vesicle compartment (vesicle SNAREs) pair with their cognate receptor complexes at the target membrane (target SNAREs), resulting in the formation of a stable core complex capable of inducing membrane fusion (10–12). It is generally thought that the function of various SNARE-associated accessory proteins provides for the necessary trafficking and regulated fusion specificity. In the case of GLUT4/IRAP-containing vesicles, the syntaxin4-SNAP23 complex appears to function as the required target SNARE, whereas VAMP2 is the predominant vesicle SNARE for insulin-stimulated GLUT4 vesicle docking and fusion (13–17). In addition, the two accessory proteins Munc18c and Synip play important functional roles in regulation of insulin-stimulated target and vesicle SNARE interactions (18–20).

Based upon this paradigm, the insulin-stimulated translocation of the GLUT4/IRAP-containing compartments must necessarily require the trafficking of these vesicles to the cell surface, subsequent to docking to and ultimate fusion with the plasma membrane. Therefore, to visualize these discrete steps in the GLUT4/IRAP translocation process, we compared the temporal relationship between GLUT4/IRAP translocation in isolated plasma membranes sheets with intact cell subcellular localization at 23 °C versus 37 °C. The data presented in this manuscript demonstrate that insulin stimulation of 3T3L1 adipocytes at 23 °C results in an accumulation of these vesicular compartments beneath the plasma membrane, which subsequently fuses slowly with the plasma membrane.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfections—Murine 3T3L1 preadipocytes were obtained from the American Type Tissue Culture repository and were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose and 10% calf serum at 37 °C in an 8% CO\(_2\) atmosphere. Confluent cultures were induced to differentiate into adipocytes as described previously (21). All studies were performed on adipocytes 8–12 days after differentiation.

The fully differentiated adipocytes were electroporated (0.16 kV and 950 microfarad) with 50 μg of the EGFP-tagged plasmids as described previously (20). Following electroporation, the adipocytes were replated on glass coverslips and allowed to recover for 30–36 h before use. Prior to insulin stimulation the differentiated adipocytes were serum-starved in Dulbecco's modified Eagle's medium containing 25 mM glucose for 2 h at either 23 °C or 37 °C.

Plasma Membrane Sheet Assay—Preparation of plasma membrane sheets from the adipocytes was performed essentially by the method of Robinson et al. (22) with minor modifications as described previously (21). Following the isolation of plasma membrane sheets, these purified membranes were used for immunoblots or indirect immunofluorescence. For immunoblotting, the sheets were directly scraped into SDS-polyacrylamide gel electrophoresis. Following electrophoresis, the amount of protein in each sample was assessed by silver staining (Silver Stain Plus Kit; Bio-Rad) and quantitated by NIH Image Analysis. The normalized samples were then separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with a polyclonal rabbit Clathrin heavy chain antibody (Transduction Laboratories), polyclonal rabbit IRAP/VP165 antibody (kindly provided by Dr. Steven Waters, Metabolix, Inc.), and polyclonal rabbit GLUT4 antibody.

For confocal immunofluorescence microscopy, the plasma membrane sheets were fixed for 20 min at 4 °C in a solution containing 2% paraformaldehyde, 70 mM KCl, 30 mM HEPES, pH 7.5, 5 mM MgCl\(_2\), and 3 mM EGTA as described previously (21). The membrane sheets were then incubated for 60 min at 25 °C with a 1:100 dilution of polyclonal rabbit GLUT4 antibody followed by incubation with a 1:50 dilution of lissamine-rhodamine-conjugated donkey anti-rabbit immunoglobulin G (IgG) (Jackson Immunoresearch Inc.) for 60 min at 25 °C.
buffered saline (PBS); pH 7.45, and were fixed and permeabilized for 10 min in ice-cold PBS containing 3.7% paraformaldehyde and 0.18% Triton X-100. After three rinses with room temperature PBS, the cells were blocked for 60 min in PBS containing 1% bovine serum albumin (Sigma) and 5% donkey serum (Sigma). The blocked cells were incubated for 60 min with 1:250–1:500 dilutions of affinity-purified rabbit lissamine-rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Inc.). Following incubation with the secondary antibodies, the cells were washed for 30 min with PBS (six changes of PBS) and subjected to GLUT4 and IRAP immunoblotting (Fig. 1B). These are representative experiments independently performed three to six times. IF, immunofluorescence; IB, immunoblotting.

RESULTS AND DISCUSSION

The Rate of Insulin-stimulated GLUT4/IRAP Translocation Is Temperature-dependent—It has been well documented that the transfer of protein cargo from one membrane compartment to another requires the trafficking, docking, and fusion of carrier vesicles with the appropriate target membrane (10–12). The rate of these transport steps are highly temperature sensitive and can be either reduced or fully inhibited depending upon specific incubation conditions (24–28). In particular, incubation of adipocytes below 19 °C has been shown to completely prevent insulin-stimulated GLUT4 translocation (29).

Therefore, we initially evaluated various conditions that would substantially reduce the rates of these processes to potentially resolve the distinct trafficking steps in GLUT4 translocation. Consistent with previous studies, we observed that at temperatures below 23 °C, there was a complete inhibition of insulin-stimulated GLUT4 translocation (data not shown). However, as observed in Fig. 1, the time-dependent increase in GLUT4 plasma membrane appearance following insulin stimulation at 23 °C (Fig. 1A, panels 1, 3, 5, 7, 9, and 11) was markedly slowed compared with that at 37 °C (Fig. 1A, panels 2, 4, 6, 8, 10, and 12). Similarly, the rate of insulin-stimulated IRAP translocation was also reduced at 23 °C (Fig. 2A, panels 1, 3, 5, 7, 9, and 11) in comparison to 3T3L1 adipocytes maintained at 37 °C (Fig. 2B, panels 2, 4, 6, 8, 10, and 12). Although the rate of insulin-stimulated translocation appeared to be reduced, the total extent of GLUT4 and IRAP protein incorporated into the plasma membrane sheets appeared to be similar as visualized by confocal immunofluorescence microscopy. To better quantify these findings, the isolated plasma membrane fraction was subjected to GLUT4 and IRAP immunoblotting (Fig. 1B and 2B). In excellent agreement with the immunofluorescence analysis, the rate but not the extent of GLUT4 and IRAP protein translocation to the plasma membrane was decreased at 23 °C compared with 37 °C. Quantitation of these data indicated that the t₁/₂ for GLUT4/IRAP translocation was ~4 min at 37 °C and increased to a t₁/₂ of ~30 min at 23 °C.

GLUT4-containing Vesicles Accumulate at the Inner Surface of the Plasma Membrane at 23 °C—There are several possible mechanisms that could account for the decreased rate of insulin-stimulated GLUT4/IRAP translocation at reduced temperatures. However, examination of the known proximal insulin signal transduction events including insulin receptor autophosphorylation, insulin receptor substrate protein tyrosine phosphorylation, stimulation of phosphatidylinositol 3-kinase activity, and, in vivo formation of phosphatidylinositol-3,4,5-trisphosphate was not significantly different between 3T3L1 adipocytes maintained at 23 °C versus 37 °C (data not shown).

Because the insulin receptor signaling cascades appeared to be fully functional, we next examined the basal and insulin-stimulated cellular distribution of GLUT4 in intact cells (Fig. 3). In the basal state, GLUT4 was predominantly localized to the perinuclear region and in small vesicles scattered throughout the cytoplasm in cells maintained at both 29 °C and 37 °C (Fig. 3, panels 1 and 2). As expected, treatment of cells at 37 °C with insulin for 15 min resulted in a clear redistribution of GLUT4 from the intracellular compartments to the plasma membrane as depicted by the appearance of a continuous rim of cell surface immunofluorescence (Fig. 3, panel 4). In the continuous presence of insulin, the translocation of GLUT4 to the plasma membrane was persistent up 90 min (Fig. 3, panels 4, 6, and 8).

In marked contrast, at 23 °C insulin stimulation for 15–30 min resulted in an accumulation of GLUT4 at the inner surface of the plasma membrane but was apparently not contiguous with the plasma membrane (Fig. 3, panels 3 and 5). This

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1 The abbreviations used are: PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein.
immunoblotting.

subjected to IRAP immunofluorescence under “Experimental Procedures” and subjected to IRAP immunofluorescence microscopy (A). The plasma membrane sheets were also detergent solubilized and subjected to Western blot analysis for IRAP (B). These are representative experiments independently performed three to six times. IF, immunofluorescence; IB, immunoblotting.

reduction occurred concomitant with a decrease in the amount of GLUT4 localized in the perinuclear region. However, at longer times of insulin stimulation at 23 °C (90 min), the plasma membrane juxtaposed GLUT4-containing vesicles appeared to decrease concomitant with the formation of a continuous rim of cell surface immunofluorescence (Fig. 3, panel 7). This occurred without any further decrease in the amount of GLUT4 localized in the small vesicular structures scattered throughout the cell cytoplasm or in the perinuclear region. In addition to GLUT4, identical results were also observed for the insulin stimulation of IRAP at 23 °C and 37 °C (data not shown). From these data, we conclude that the insulin-stimulated trafficking (movement) of the GLUT4/IRAP-containing vesicles to the plasma membrane was not significantly affected at 23 °C compared with 37 °C but that either the docking (plasma membrane binding) or fusion (physical integration into the plasma membrane) was impaired at the lower temperature.

Expressed GLUT4-EGFP and EGFP-IRAP Fusion Proteins Are Appropriately Localized in 3T3L1 Adipocytes—To further examine the plasma membrane association of the GLUT4/IRAP-containing vesicles, we prepared GLUT4-EGFP and EGFP-IRAP fusion proteins to increase the resolution of these structures by confocal fluorescence microscopy. However, to ensure that the expression of these fusion proteins was localized to the appropriate intracellular compartments and underwent the proper degree of insulin-stimulated translocation, we first compared the subcellular localization of the expressed GLUT4-EGFP protein with endogenous IRAP (Fig. 4A). In the basal state, both the expressed GLUT4-EGFP and endogenous IRAP proteins were localized in small vesicular compartments and in the perinuclear region (Fig. 4A, panels 1 and 3). As expected, there was a substantial degree of co-localization as observed by the appearance of yellow in the merged image (Fig. 4A, panel 5). Insulin stimulation resulted in the formation of cell surface GLUT4-EGFP and endogenous IRAP, again with substantial co-localization (Fig. 4A, panels 2, 4, and 6). Similarly, expression of EGFP-IRAP resulted in an identical basal state distribution as the endogenous GLUT4 protein (Fig. 4B, panels 1, 3, and 5). Furthermore, following insulin stimulation both the expressed EGFP-IRAP and endogenous GLUT4 displayed the identical degree of plasma membrane translocation (Fig. 4B, panels 2, 4, and 6). Thus, these data demonstrate that both our GLUT4-EGFP and EGFP-IRAP fusion constructs are correctly localized and display the appropriate insulin-stimulated trafficking when expressed in 3T3L1 adipocytes.

Reduced Temperature Decreases the Plasma Membrane Fusion of Docked GLUT4-EGFP-containing Vesicles—Having demonstrated the fidelity of these fusion proteins, we next examined the effect of temperature on the translocation of GLUT4-EGFP (Fig. 5). To more closely compare the relative distribution of the GLUT4/IRAP-containing vesicles with the plasma membrane, the cell surface was also subjected to biotinylation followed by strepavidin-Texas Red labeling. As previously observed for endogenous GLUT4 (Fig. 4), insulin stimulation for 30 min at 23 °C resulted in an accumulation of a discontinuous punctate distribution of GLUT4-EGFP beneath the plasma membrane (Fig. 5A, panel 1). This is more clearly visualized when a segment of the plasma membrane is magnified (Fig. 5A, panel 1, inset). In contrast, insulin stimulation for 30 min at 37 °C resulted in a continuous plasma membrane distribution of GLUT4-EGFP that was clearly incorporated into the plasma membrane as depicted by the overlap between the GLUT4-EGFP and strepavidin-Texas Red signals (Fig. 5A, panel 2). Quantitation of the number of cells displaying these
two phenotypes demonstrated that at 37 °C, there was a rapid formation of cells displaying a continuous surface labeling (Fig. 5A). In contrast, insulin stimulation at 23 °C resulted in a large increase in the number of cells with a subplasma membrane punctate distribution that slowly converted to a smooth continuous cell surface rim fluorescence. Essentially identical results were also obtained when we examined the temperature and time dependence of insulin-stimulated EGFP-IRAP subcellular localization (data not shown). Thus, the accumulation of GLUT4/IRAP-containing vesicles beneath the cell surface at 23 °C strongly suggests that the plasma membrane fusion of these vesicles was decreased with relatively little effect on plasma membrane docking.

In conclusion, we have taken advantage of reduced temperature to decrease the overall rate of insulin-stimulated GLUT4/IRAP vesicle translocation to the plasma membrane. Incubation of 3T3L1 adipocytes at 23 °C resulted in an approximate 7-fold increase in the t1/2 for insulin-stimulated appearance of GLUT4/IRAP in the plasma membrane without any major effect on the extent of translocation. Importantly, reduced tem-
temperature did not have any significant effect on the activation of the insulin receptor kinase, insulin receptor substrate tyrosine phosphorylation, and phosphatidylinositol 3-kinase activation or generation and/or localization of phosphatidylinositol-3,4,5-trisphosphate in intact 3T3L1 adipocytes. Instead, rather than a rapid insulin-stimulated plasma membrane fusion of the GLUT4/IRAP-containing vesicles, they were observed to accumulate adjacent to the inner surface of the plasma membrane. Based on the resolution limit of the confocal microscope, the average center of the GLUT4-EGFP signal appeared localized within 140 nm of the average center of the biotin-streptavidin-Texas Red signal. Although this separation should only be considered as a rough estimate, this dimension is consistent with the GLUT4/IRAP vesicles trapped in a docked state that can only then slowly integrate into the plasma membrane lipid bilayer. Thus, the simplest and most direct model accounting for these data is that insulin stimulation resulted in a normal trafficking and docking of the GLUT4/IRAP-containing vesicles with the plasma membrane at 23 °C. However, at this temperature the rate of vesicle fusion with the plasma membrane was decreased accounting for the overall reduction in the time course of GLUT4/IRAP translocation and appearance of the abutting GLUT4/IRAP-containing vesicles.

Although less likely, there are several alternative explanations for these observed data. For example, the apparent punctate pattern of GLUT4 accumulation beneath the cell surface may represent a clustering of vesicles in discrete plasma membrane subdomains that are inaccessible to biotin-streptavidin-Texas Red cell surface labeling. At 23 °C the fluidity of the plasma membrane or vesicle lipid bilayer may also be substantially reduced, thereby lowering the rate of protein mixing. Under such conditions, it remains possible that the GLUT4 vesicles are actually fused with the plasma membrane but have not yet undergone lateral protein diffusion. On the other hand, the accumulation of these vesicles may not actually be juxtaposed to the plasma membrane and could represent a nexus point for progression to the plasma membrane. Additional studies at the electron microscopic level are now required to provide the necessary resolution to address these issues. In any case, the ability to distinguish between GLUT4/IRAP vesicle trafficking, docking, and fusion will provide an important experimental tool to further our understanding of the mechanisms regulating GLUT4 translocation.

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