Characterization of the Insulin-regulated Membrane Aminopeptidase in 3T3-L1 Adipocytes*

Stuart A. Ross, Hazel M. Scott, Nicholas J. Morris, Wai-Yee Leung, Fei Mao, Michael E. Lienhard, and Susanna R. Keller§

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755 and Molecular Probes, Inc., Eugene, Oregon 97402

A novel membrane aminopeptidase has been identified as a major protein in vesicles from rat adipocytes containing the glucose transporter isotype Glut4. In this study we have characterized this aminopeptidase, referred to as vp165, in 3T3-L1 adipocytes. The subcellular distributions of vp165 and Glut4 were determined by immunoisolation of vesicles with antibodies against both proteins, by immunofluorescence, and by subcellular fractionation and immunoblotting. Relative amounts of vp165 at the cell surface in basal and insulin-treated cells were assayed by cell surface biotinylation. These experiments showed that vp165 and Glut4 were entirely colocalized and that vp165 increased markedly at the cell surface in response to insulin, in a way similar to Glut4. When intact cells were assayed with a novel, membrane-impermeant fluorogenic substrate for vp165, we found that insulin stimulated aminopeptidase activity at the cell surface. This observation provides direct evidence for the functional consequence of vp165 translocation.

An important effect of insulin is to increase glucose transport into muscle and fat cells. The basis of this effect is an increase in the amount of the glucose transporter isotype Glut4 in the plasma membrane, which is probably largely due to insulin-stimulated fusion of intracellular vesicles containing Glut4 with the plasma membrane (1, 2). We and others have developed methods for isolating these Glut4 vesicles from fat and muscle cells and are analyzing the proteins in them (3–6). A major protein, of 165 kDa, in the Glut4 vesicles from rat adipocytes (designated vp165) has recently been characterized by the Pilch laboratory and ourselves (3, 7, 8). Through cloning of the cDNA for vp165, we found that it is a novel membrane aminopeptidase, consisting of a 109 residue cytoplasmic amino-terminal domain that contains several potential sorting signals similar to those in Glut4, a single transmembrane segment, and a large lumenal domain that contains the active site (9).

The distribution of vp165 in rat adipocytes has been determined for basal and insulin-treated cells by subcellular fractionation and immunoblotting (3, 7). These earlier studies showed that vp165, like Glut4, is concentrated in the low density microsomes and redistributes to the plasma membrane in response to insulin. Moreover, they showed that intracellular vp165 is located in vesicles that also contain Glut4, since immunoassay of vesicles with antibodies against Glut4 also adsorbed most of the vp165 (3, 7). However, these earlier studies did not rigorously address the question of whether vp165 and Glut4 are entirely colocalized and translocate in a quantitatively similar way in response to insulin. One reason for this is that subcellular fractionation provides only a crude indication of subcellular localization and typically underestimates translocation due to contamination of the plasma membrane fraction with intracellular membranes (see Results and Discussion). Also, since at the time antibodies that immunoadsorbed vp165 were not available, it was not possible to perform the complementary immunoassay of vesicles with antibodies against vp165, in order to determine whether all the Glut4 is in vesicles that also contain vp165. Another question not addressed by these earlier studies, which is especially important now that vp165 has been established to be an aminopeptidase, is whether the translocation of vp165 to the plasma membrane is, in fact, accompanied by the appearance of cell surface aminopeptidase activity.

In the present study, we have characterized vp165 in 3T3-L1 adipocytes, cultured cells that are very insulin-responsive and have been employed extensively for the investigation of insulin-stimulated Glut4 translocation, as well as other insulin actions. Through the use of better antibodies against vp165 and a membrane-impermeant aminopeptidase substrate, we have been able to answer the questions posed above.

EXPERIMENTAL PROCEDURES

Antibodies—The antibodies against vp165 and Glut4 used throughout were affinity-purified rabbit antibodies against the entire intracellular domain of vp165 and the carboxyl-terminal 19 amino acids of Glut4, unless noted otherwise. These have been characterized previously (9, 10).

Cell Culture—3T3-L1 fibroblasts were cultured and differentiated into adipocytes as described previously (11). For immunofluorescence, the cells were grown on glass coverslips in 6-well plates. Before use, cells were put into serum-free Dulbecco’s modified Eagle’s medium for 2 h. Cells were treated with insulin in this medium, unless stated otherwise.

Isolation of Glut4 Vesicles—This procedure is the one previously described in detail (10, 12). Plates (10 cm) of adipocytes were treated with 160 nM insulin for 15 min or left in the basal state. Each plate was homogenized in 8 ml 150 mM KCl, 2 mM MgCl₂, 20 mM Hepes, pH 7.2, and the homogenate was centrifuged at 16,000 × gmax for 20 min. The supernatant, which contains the microsomes and cytosol, was used for the adsorption of vesicles. Aliquots (1.3 ml) were incubated with antibodies against Glut4 or vp165, or with irrelevant rabbit immunoglobulin (Sigma), bound to protein A on the surface of formaldehyde-fixed Staphylococcus aureus cells (11 μg antibodies on 4 μl of S. aureus cells), for 2 h at 4 °C. The S. aureus cells were collected by centrifugation and washed, and the vesicle proteins were eluted with SDS sample buffer containing 8 M urea.

Subcellular Fractions—Samples of four separate preparations of plasma membranes and low density microsomes, isolated from basal...
and insulin-treated (1 μl for 20 min) 3T3-L1 adipocytes according to the method of Piper et al. (13), were generously provided by Drs. Harry Heimberg and Mike Mueckler, Washington University School of Medicine.

Cell Surface Biotination—3T3-L1 adipocytes in 6-well plates were treated with 160 μM insulin for 15 min or left in the basal state. The cells were then resuspended (1 ml) in 1% bovine serum albumin (BSA), 50 mM NaCl, 1.25 mM CaCl2, 1.25 mM MgSO4, 5 mM sodium phosphate) containing 20 mg/ml Hepes, pH 7.4, and then treated with 1 ml of 1 mM sulfo-NHS-LC-biotin (Pierce) in this buffer with constant shaking for 30 min at 4°C. The cells were washed three times with ice-cold 25 mM ethanalamine, 150 mM NaCl, 20 mM Tris-Cl, pH 7.4, in order to quench any non-specific biotinylation, and then lysed in 1 ml/well with 1% deoxy-ene glycol dodecyl ether, 150 mM NaCl, 20 mM Tris-Cl, pH 7.4, plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml EP475, 10 μg/ml leupeptin, 1 μg/ml pepstatin A). The lysate was centrifuged at 16,000 × g max. for 20 min at 4°C. Aliquots of the supernatant (0.4 ml) were immunoprecipitated at 4°C by incubation with 10 μg of antibodies against vp165 or Glut4 or with 10 μg of irrelevant rabbit IgG for 2 h, followed by collection on 10 μl of protein A-Sepharose (Pharmacia Biotech Inc.) for 1.5 h. SDS samples of the immunoadsorbrates were separated by gel electrophoresis and blotted with streptavidin-horseradish peroxidase (see below). Control experiments, in which samples of the initial supernatant, the supernatant after immunoprecipitation, and the immunoprecipitate were run in parallel, showed that over 90% of the vp165 was immunoprecipitated and that the amount of vp165 in the immunoprecipitates from the basal and insulin-treated cells was the same.

Tyrosine Phosphorylation—Cells (10-cm plates) were treated with 160 μM insulin for various times and lysed in 2 ml/plate of 1.5% Nonidet P-40, 10% glycerol, 150 mM NaCl, 40 mM Hepes, pH 7.5, containing phosphatase inhibitors (10 mM sodium pyrophosphate, 10 mM NaF, 2 mM sodium vanadate) and protease inhibitors (see above). Aliquots of the lysate (0.5 ml) were immunoprecipitated with 10 μg of antibodies against vp165 or Glut4 or with irrelevant rabbit immunoglobulin, as described above. SDS samples of the lysates, lysates after immunoprecipitation, and immunoprecipitates were blotted for phosphotyrosine and also vp165 and Glut4 (see below). Over 90% of both vp165 and Glut4 was immunoprecipitated under these conditions.

Immunoblotting—Polypeptides were separated by SDS-gel electrophoresis and transferred to Immobilon-P membranes (Millipore) for 3 h at 300 mA. The membrane was blocked with 5% nonfat dry milk in 150 mM NaCl, 20 mM Tris-Cl, pH 7.4 (TBS), treated with antibodies against vp165 or Glut4 at 5 μg/ml in 1% milk, TBS, with 0.1% Tween 20, TBS, treated with goat antibodies against rabbit immunoglobulin coupled to horseradish peroxidase (Life Technologies, Inc., 1/3000 dilution) in 1% milk, TBS, and then washed with 0.1% Tween 20, TBS followed by TBS. In the case of the blots for biotin and phosphotyrosine, milk was replaced by bovine serum albumin (BSA), and the binding protein was streptavidin-horseradish peroxidase (Pierce; 1 μg/ml) and RC20-horseradish peroxidase (Transduction Labs, 0.1 μg/ml). Autoradiograms were scanned on an ISCOM scanner (Molecular Dynamics, Sunnyvale, CA), and the bands were quantitated with the software program (ImageQuant, Molecular Dynamics). In all cases detection was with the enhanced chemiluminescence reagent (Amersham Corp.).

Immunofluorescence—Cells on coverslips were washed with KRBP, 1 mM BSA, treated with 160 μM insulin for 15 min in this buffer, or left in the basal state. The cells were then fixed with 3.5% paraformalde-hyde for 5 min, permeabilized with 0.2% saponin in 1% BSA, TBS for 5 min, and treated with rabbit antibodies against vp165 and a mouse monoclonal antibody against Glut4 (Genzyme), each at 5 μg/ml, in 1% BSA, TBS for 30 min. The cells were washed with 1% BSA, TBS, for 5 min, incubated with goat antibodies against rabbit immunoglobulin conjugated to fluorescein and horse antibodies against mouse immunoglobulin (conjugated to Texas Red; Vector Laboratories), both at 6 μg/ml, together with the DNA stain DAPI, at 0.4 μg/ml, in 1% BSA, TBS for 30 min in the dark, washed with 1% BSA, TBS, mounted in a drop of fluorescein isothiocyanate-Guard (Testog), and viewed with the appropriate filters in a Nikon Optiphot 2 microscope equipped for epifluorescence.

Aminopeptidase Activity—Aminoacyl derivatives of 7-amino-4-methylcoumarin (AMC) are fluorogenic substrates for aminopeptidases (En-zyme Systems Products). We synthesized the L-lysyl derivative conjugated via the methylene group at position 4 to the sulfhydryl of glutathione (designated Lys-AMC-glutathione). The details of the synthesis are available on request, and the compound is now commercially available from Molecular Probes. The compound gave the expected nmr and elemental analyses. When solutions (0.5 mM) of this compound were in the beam of the spectrophotometer without enzyme, there was a relatively slow increase in fluorescence that virtually ceased at a low value after about 30 min. We attribute this fluorescence increase to a trace of photolabel impurity. Because of it, when the enzyme assay was performed in the spectrophotometer (see below and Fig. 4B), the substrate was first left in the beam without enzyme for about 45 min; and when the enzyme assay was performed by measuring the fluorescence of aliquots taken from an assay mixture at timed intervals (see below and Fig. 5), the fluorescence was read immediately upon placing the aliquot in the spectrophotometer.

The membrane impermeability of the Lys-AMC-glutathione was tested with low density microsomes, freshly prepared (not frozen) from rat adipocytes as described in Keller et al. (9), except for the omission of EDTA in the buffer. Activity in the absence and presence of a nonionic detergent was assayed at 25°C in a recording fluorescence spectrometer (Hitachi model F-3010) with excitation at 365 nm and emission at 455 nm in a 0.5-ml cuvette with 5-mm path length.

For the assay of the aminopeptidase activity of intact 3T3-L1 adipocytes, the serum-free medium on 6-well plates was replaced with KRBP (1.4 ml/well), and the cells were either treated with 300 nM insulin for 15 min or left in the basal state, at 37°C. Lysyl-AMC-glutathione was then added from a stock solution, such that the final concentration and concentration in the absence of enzyme, the fluorescence increase was 0.5 μM. At various times aliquots (300 μl) were removed from each well, diluted with 400 μl of KRBP, and kept on ice until fluorescence was measured, as described above.

RESULTS AND DISCUSSION

Colocalization of vp165 with Glut4 in Intracellular Vesicles—Intracellular vesicles containing Glut4 can be isolated from the microsomal fraction of 3T3-L1 adipocytes by immunoadsoption with antibodies against the carboxyl terminus of Glut4 attached to protein A on the surface of formaldehyde-fixed S. aurasus cells (10). Using this approach, we adsorbed vesicles from the microsomal fraction of basal and insulin-treated 3T3-L1 adipocytes with antibodies against vp165 and Glut4. The adsorbed and nonadsorbed fractions were analyzed for vp165 and Glut4 by immunoblotting (Fig. 1A). With microsomes from both basal and insulin-treated cells, virtually all the vp165 was adsorbed with the antibodies against Glut4, and conversely, virtually all the Glut4 was adsorbed with the antibodies against vp165. Thus, for cells in both the basal and insulin-treated state, all vesicles containing Glut4 contain vp165, and vice versa. The less intense signals for vp165 and Glut4 in the samples from insulin-treated cells is due to the translocation of a portion of each protein from the microsomes to the plasma membranes (see below). A potential alternative explanation of these results, cross-reaction of the antibodies, is very unlikely, since in vp165, in a detergent lysate of 3T3-L1 adipocytes was not immunoprecipitated or immunoblotted by the anti-Glut4, and the same was the case for Glut4 and the anti-vp165 (data not shown).

The subcellular distributions of vp165 and Glut4 were also compared by double-label immunofluorescence (Fig. 1B). The patterns of staining for the two proteins were superimposable, with the most intense staining in vesicles surrounding a portion of the nucleus and additional staining in vesicles throughout the cell. This distribution is the same as the one previously described for Glut4 (13).

Insulin-stimulated Translocation of vp165 to the Plasma Membrane—In one approach to test the translocation of vp165 to the plasma membrane in response to insulin, the plasma membrane and low density microsomal fractions from basal and insulin-treated 3T3-L1 adipocytes were analyzed for vp165 and Glut4 by immunoblotting (Fig. 2). The level of vp165 and Glut4 in the plasma membranes increased about 2-fold in response to insulin, with a concomitant reduction in the low density microsomes to about one-half the basal level. Thus, in this assay the insulin-elicited redistribution of vp165 was quantitatively the same as that of Glut4.

The abbreviations used are: BSA, bovine serum albumin; AMC, 7-amino-4-methylcoumarin; DAPI, 4,6-diamidino-2-phenylindole.
Previous studies have indicated that the measurement of Glut4 translocation in 3T3-L1 adipocytes by subcellular fractionation and immunoblotting substantially underestimates the magnitude of the effect, probably because the plasma membrane fraction is contaminated by some intracellular vesicles containing Glut4. In our hands and others, the increase in Glut4 at the cell surface, when assessed by photoaffinity labeling, is 12–17-fold (10, 14). This increase corresponds more closely to the increase in glucose transport, which is typically 10–20-fold (10, 14). This criticism would also apply to the measurement of vp165 translocation by immunoblotting the plasma membrane fractions, and we were therefore led to develop a cell surface biotinylation method for assessment of vp165 translocation. This method was based on the finding, made by Kandror and Pilch (15), that vp165 at the cell surface of rat adipocytes is susceptible to biotinylation.

Basal and insulin-treated 3T3-L1 adipocytes were cooled to 4°C, in order to prevent membrane trafficking (16), and then reacted with a membrane-impermeant reagent that biotinylates exposed amino groups. Subsequently vp165 was isolated by immunoprecipitation and its extent of biotinylation measured by blotting with streptavidin conjugated to horseradish peroxidase. The results in Fig. 3 show that by this assay the increase in vp165 at the cell surface in response to insulin was approximately 8-fold (compare lane 1, with lanes 2–5). This higher value is closer to that expected on the basis of the results with Glut4 described above. In several of these experiments we also isolated Glut4 by immunoprecipitation and examined it for biotinylation, but did not detect any biotinylation (data not shown). This outcome is explicable by the fact that Glut4 has only one lysine in its predicted extracellular domain, whereas vp165 has 45 lysines in its extracellular domain (9, 17). In the future, it should be possible to determine the kinetics of vp165 recycling in basal and insulin 3T3-L1 adipocytes by use of this cell surface biotinylation method, in an analogous way to the determination of the kinetics of Glut4 recycling by photoaffinity labeling Glut4 at the cell surface (18).

Aminopeptidase Activity at the Cell Surface—Since vp165 increases markedly at the cell surface in response to insulin, we would expect insulin to increase aminopeptidase activity at the cell surface. In order to test this possibility, we synthesized the highly charged, fluorogenic substrate, Lysyl-AMC-gluthathione, which was expected to be membrane-impermeant (Fig. 4A). Previously we found that purified vp165 was most active toward leucyl and lysyl substrates (9). In order to establish that the substrate was membrane-impermeant, we assayed it with intact and detergent-solubilized low density microsomes from rat adipocytes. We have found by proteolytic analysis that the vp165 in this fraction is in sealed Glut4 vesicles with its active site in the lumen (9) and have also found by immunoprecipitation that at least 90% of the activity against this substrate in the low density microsomes is due to vp165 (data not shown). As shown in Fig. 4B, the activity toward the lysyl-AMC-gluthathione increased at least 15-fold upon solubilization of the membranes with a nonionic detergent. This result demonstrates that the substrate is not readily membrane permeant and provides further evidence that the active site of the enzyme is lumenal.
It seemed possible that the trafficking of vp165 and Glut4 upon differentiation—

the only one to date that is so similar to Glut4 in distribution (9). We determined the relative amounts of vp165 and of Glut4 during differentiation of 3T3-L1 fibroblasts into adipocytes by immunoblotting (Fig. 6). vp165 was present in the fibroblasts, and its level increased approximately 6-fold during differentiation, with the largest increase occurring between days 2 and 4 after the initiation of differentiation. By contrast, and in agreement with results in the literature (23, 24), Glut4 was not detectable in fibroblasts and increased most markedly between days 6 and 8 after the initiation of differentiation. Thus, the control of vp165 expression in 3T3-L1 cells differs substantially from that of Glut4.

Conclusions—The results herein indicate that in 3T3-L1 adipocytes vp165 and Glut4 have identical subcellular distributions and translocate to the cell surface to the same marked extent in response to insulin. Although a number of other proteins in Glut4 vesicles have been identified (25), vp165 is the only one to date that is so similar to Glut4 in distribution and insulin-stimulated translocation. This situation is presumably due to the presence of similar motifs controlling the trafficking of these two proteins (9).

The translocation of vp165 was accompanied by the pre-

2 This value is based upon an estimate of the ng vp165 per mg protein in the low density microsomes from rat adipocytes (3) and the relative intensities of vp165 in known amounts of rat adipocyte low density microsomes and 3T3-L1 adipocyte lysates on an immunoblot.
predicted appearance of cell surface aminopeptidase activity. Thus, the characterization of this vesicle protein has led to the discovery of a hitherto unknown insulin effect on cell surface function. In order to understand the ramifications of this effect, it is now important to identify the physiological substrates for vp165. Finally, it should be noted that the Glut4 vesicles contain a number of unidentified major proteins (3–5), and so it is possible that there are other proteins, like Glut4 and vp165, that translocate markedly in response to insulin and modify functions at the cell surface.

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Fig. 6. Expression of vp165 and Glut4 upon differentiation of 3T3-L1 adipocytes. SDS samples were prepared from 10-cm plates of 3T3-L1 cells 2 days after reaching confluence (day 0 for differentiation) and at various days thereafter during the differentiation protocol. Samples containing 25 µg of protein were immunblotted for vp165 and Glut4 (lanes 1–6). The standards (lanes 7–13) contain the stated fraction of 25 µg of protein of the day 8 sample. A repetition of this entire experiment yielded similar results.