The Amino Terminus of Insulin-responsive Aminopeptidase Causes Glut4 Translocation in 3T3-L1 Adipocytes*

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Steven B. Waters, Matt D’Auria, Stuart S. Martin‡, Chris Nguyen, Lynn M. Kozma, and Kenneth L. Luskey§

From Metabolex, Inc., Hayward, California 94545 and the Department of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, California 92039

The insulin-responsive aminopeptidase (IRAP) is a constituent of the vesicles that contain the insulin-regulated glucose transporter (Glut4). Like Glut4, IRAP translocates to the cell surface in response to insulin. Microinjection into 3T3-L1 adipocytes of a glutathione S-transferase (GST) fusion protein containing the cytosolic portion of IRAP (GST-IRAP-(1-109)), resulted in translocation of Glut4 to the cell surface. Immunostaining of 3T3-L1 adipocytes for Glut4 showed that the percentage of cells with substantial cell surface Glut4 was 10% in unstimulated cells, 8% following injection of GST, and 27% following injection of GST-IRAP-(1-109). Increased cell surface Glut4 occurred within 5-10 min following injection and was maintained for at least 4 h. A fusion protein containing only 28 amino acids from IRAP (GST-IRAP-(55-82)) was as effective in increasing cell surface Glut4 as stimulation with 100 nM insulin (44% versus 43%, respectively). In contrast to insulin-stimulated Glut4 translocation, the redistribution of Glut4 following injection of GST-IRAP-(55-82) was not blocked by wortmannin or co-injection with a SH2 domain from the regulatory subunit of phosphatidylinositol 3-kinase. These data suggest that the amino terminus of IRAP interacts with a retention/sorting protein that also regulates the distribution of Glut4 in insulin-responsive cells.

Translocation of intracellular vesicles containing the insulin-responsive glucose transporter Glut4 to the cell surface is one of the most important effects of insulin in adipose and skeletal muscle tissues (1-4). In the absence of insulin, approximately 95% of cellular Glut4 is sequestered in an intracellular compartment. The carboxyl terminus of Glut4, which projects into the cytoplasm of the cell, appears to be a critical region involved in this process. When chimeric transporters of Glut1 and Glut4 were examined, substitution of the carboxyl-terminal 30 amino acids of Glut1 with the corresponding Glut4 sequence results in sequestration of the chimera in an intracellular compartment (5-7), indicating that this region functions in the intracellular trafficking and retention of Glut4. Several studies have suggested that a dileucine motif in the carboxyl terminus of Glut4 has a significant role in this process (8-10).

Studies of immunoadsorbed Glut4 vesicles have identified a number of other proteins in these vesicles. One of these is insulin-responsive aminopeptidase (IRAP),1 a zinc-dependent aminopeptidase (11-13) that is oriented in the vesicle membrane with its 109-amino acid amino-terminal end projecting into the cytoplasm, a single transmembrane domain, and a large catalytic domain within the lumen of the vesicle. IRAP has also been called gp160 and vp165. The distribution of IRAP within adipocytes is very similar to that of Glut4. In the basal state the majority of IRAP is sequestered in an intracellular compartment, and following stimulation with insulin both Glut4 and IRAP are translocated to the cell surface (14, 15). Interestingly, the amino terminus of IRAP contains two dileucine motifs and several acidic regions similar to those that occur in Glut4. The similarity between the carboxyl terminus of Glut4 and the amino terminus of IRAP suggests that these two molecules are sorted within the cell by a common mechanism.

To test the hypothesis that an intracellular sorting mechanism exists which recognizes both Glut4 and IRAP, we used microinjection to introduce the cytoplasmic domain of IRAP into 3T3-L1 adipocytes and examined its effect on the cellular distribution of Glut4. We found that introduction of this region of IRAP into 3T3-L1 adipocytes resulted in rapid and sustained translocation of Glut4 from an intracellular compartment to the cell surface. In contrast to the action of insulin, Glut4 translocation following microinjection of IRAP was independent of phosphatidylinositol 3-kinase activation, suggesting that it acts downstream of this signaling element. By deletion analysis, we identified a 28-amino acid region of IRAP, which was sufficient to cause Glut4 translocation.

EXPERIMENTAL PROCEDURES

Materials—1F8, a monoclonal Glut4 antibody with an epitope in the carboxyl-terminal 30 amino acids (16) was from Charles River-East Acres Biologicals (Southbridge, MA). Anti-glutathione S-transferase antibody was a gift of Dr. Mike Czech (University of Massachusetts Medical School, Worcester, MA). The fluorochrome-conjugated antibodies, anti-mouse IgG-dichlorotriazinylamino fluorescein and anti-rabbit IgG-AMCA were from Jackson ImmunoResearch (West Grove, PA). Tetramethylrhodamine-conjugated phalloidin was from Sigma.

GST-IRAP Fusion Proteins—Amino acids 1-109 of rat IRAP (GenBank: accession number U32990) was subcloned from a rat brain cDNA library using 5’-cgggaatctgatcataactttaccaatgat-3’ and 5’-cggaattcgcgttggcagaggggtaactgta-3’ as primers for the polymerase chain reaction reaction. The resulting polymerase chain reaction product was verified by sequencing of both strands and inserted into pGEX-5X (Pharmacia Biotech Inc.) using the BamHI and EcoRI sites within the primers. All the deletion constructs were prepared using the same method with appropriate oligonucleotide primers. The GST control was produced from pGEX-5X without an insert. GST proteins were expressed in Escherichia coli by induction with 100 mM isopropylthiogalactopyranos}

1 The abbreviations used are: IRAP, insulin-responsive aminopeptidase; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; Glut1, glucose transporter 1; Glut4, glucose transporter 4; GST, glutathione S-transferase; PI3K, phosphatidylinositol 3-kinase; SH2 domain, Src-homology 2 domain.
Fetal bovine serum. Coverslips were incubated 1 h with a fluorescein-conjugated anti-rabbit IgG to identify injected protein being assessed. The independent determinations of the two observers were always within 5% for the number of cells scored positive, and the average of the two scores was used. Membrane Ruffling—Formaldehyde-fixed cells (3.7% formaldehyde, 10 min) were washed in PBS for 10 min and then permeabilized with 0.1% Triton X-100 in PBS for 8 min. Polymerized actin was stained by incubating coverslips with 0.5 µg/ml phalloidin-tetramethylrhodamine in PBS and injected cells identified with fluorescein-conjugated anti-sheep IgG. Cells with fluorescein staining in the cytoplasm were scored for the presence of actin aggregates at the cell periphery (membrane ruffles).

RESULTS

Incubation of 3T3-L1 adipocytes with insulin results in translocation of Glut4 from an intracellular vesicular compartment to the plasma membrane. Translocation of Glut4 can be determined in single cells (17) and is observed as an increase in the percentage of adipocytes with Glut4 immunostaining at the plasma membrane. 3T3-L1 adipocytes were incubated with and without 100 nM insulin for 30 min at 37 °C, immunostained for Glut4, and the percentage of cells with substantial cell surface Glut4 immunostaining was determined. Average values from nine experiments for unstimulated adipocytes were 6.8% ± 1.0% (mean ± S.E.) and 44.2% ± 3.2% following insulin stimulation.

Images of cells microinjected with GST fusion proteins are shown in Fig. 1. Adipocytes were immunostained with an anti-GST antibody and an anti-rabbit-AMCA conjugate to mark injected cells (A and C) or anti-Glut4 and an anti-mouse-fluorescein conjugate to determine Glut4 distribution (B and D). Injection of adipocytes with GST alone did not result in Glut4 translocation to the cell surface (Fig. 1B). In contrast, injection of a GST fusion protein containing amino acids 1–109 of IRAP (GST-IRAP-(1–109)) led to translocation of Glut4 to the plasma membrane (Fig. 1, C and D). Quantification of this effect is shown in Fig. 2. Only 8% of cells were positive for plasma membrane staining of Glut4 after microinjection of GST alone, whereas GST-IRAP-(1–109) resulted in a significant increase to 27% of the cells (p < 0.01).

We were interested in identifying a more defined region of the amino terminus of IRAP that retained the ability to cause translocation of Glut4 to the cell surface. A series of deletion constructs were prepared and microinjected into adipocytes to determine their activity (Fig. 2). IRAP-(35–59) and IRAP-(78–109) had no effect on the subcellular distribution of Glut4. In contrast, IRAP-(1–52) and IRAP-(55–82) caused translocation...
of Glut4 to the cell surface. IRAP-(55–82) had the highest activity, which was comparable with that following stimulation with 100 nM insulin. We have also found that injection of a synthetic peptide corresponding to IRAP-(55–82) is effective in inducing Glut4 translocation (data not shown).

In addition to Glut4 translocation, insulin stimulation of 3T3-L1 adipocytes also causes rearrangement of actin resulting in plasma membrane ruffling (18). This can be observed in cells by staining F-actin with phalloidin. We injected GST-IRAP-(1–109) and GST-IRAP-(55–82) to see if they caused plasma membrane ruffling (Fig. 3). Treatment with insulin increased the percentage of cells with plasma membrane ruffling from 10% in unstimulated cells to 55% following a 10-min incubation with 100 nM insulin. However, injection of GST-IRAP-(1–109) or GST-IRAP-(55–82) had no effect on plasma membrane ruffling.

There are several possible mechanisms by which injection of IRAP into 3T3-L1 adipocytes might cause an increase in Glut4 content at the cell surface. Since Glut4 continuously recycles between an intracellular compartment and the cell surface, inhibiting internalization would lead to an increase in plasma membrane Glut4 content. Alternatively, injection of Glut4 might saturate an intracellular retention mechanism which would release Glut4 to the cell surface. These two mechanisms would be expected to have very different kinetics. It’s estimated that a 20-fold increase in cell surface Glut4 is achieved with a t1/2 of 138 min following a 40-fold decrease in the rate of endocytosis as compared with a t1/2 of 3.5 min for 40-fold increase in the rate of Glut4 exocytosis (19).

To assess changes occurring over relatively short time periods, we plated the adipocytes on coverslips with grids such that the time of injection and the location could be recorded. Microinjection of 20 mg/ml GST-IRAP-(55–82) caused an increase in cell surface Glut4 within 5–9 min after injection (Table I). The effect of GST-IRAP-(55–82) on Glut4 translocation was maximal within 5 min and persisted for 4 h. Injection of 20 mg/ml GST had no effect at any of these times (data not shown). The effect of the GST-IRAP concentration on Glut4 translocation is shown in Table I. A progressive increase in Glut4 translocation was observed from 1–10 mg/ml GST-IRAP-(55–82) with a maximal effect at 10 mg/ml, suggesting that the response is dependent on concentration. Based on the estimated volume of injection and the concentration, we estimate that this effect occurs with 2–20 million molecules/cell. As the estimated number of Glut4 molecules per 3T3-L1 cell is 280,000 (20), a 10–

![Fig. 2](image-url)  
**FIG. 2. Effect of microinjecting GST-IRAP fusion proteins on Glut4 translocation.** 3T3-L1 adipocytes were incubated in serum-free medium for 2 h and microinjected with either 15 mg/ml GST or a GST fusion protein consisting of different portions of the amino terminus of IRAP. Two hours after microinjection, cells were fixed and immunostained with anti-GST and anti-Glut4 (1F8) as described under “Experimental Procedures.” The percentage of microinjected adipocytes with substantial plasma membrane staining for Glut4 was determined. Between 100 and 900 injected cells were used in the analysis of each fusion protein. Fusion proteins that significantly increased Glut4 translocation versus injection of GST alone are indicated with an asterisk (p ≤ 0.01). The mean ± S.E. from four independent experiments is shown. For comparison, the percentage of adipocytes with substantial cell surface Glut4 staining before and after insulin stimulation (100 nM insulin, 30 min) is shown.

![Fig. 3](image-url)  
**FIG. 3. Effect of insulin and GST-IRAP fusion proteins on membrane ruffling.** Prior to microinjection, 3T3-L1 adipocytes were incubated in serum-free medium for 12–16 h. Individual cells were microinjected with 10 mg/ml sheep IgG (Control and Insulin) or with 10 mg/ml sheep IgG and either 15 mg/ml GST-IRAP-(1–109) or 20 mg/ml GST-IRAP-(55–82). Thirty minutes following injection, 3T3-L1 adipocytes were incubated in medium alone (Control, IRAP-injected cells) or with 100 nM insulin for 10 min. Cells were fixed with 3.7% formaldehyde and immunostained with anti-sheep IgG conjugated to fluorescein to detect injected cells and phalloidin conjugated to rhodamine to stain polymerized actin. The percentage of injected adipocytes with actin membrane ruffles was determined. Between 200 and 500 injected cells were used in the analysis. The mean ± S.D. from three experiments is shown.

| Time after injection | Glut4 translocation |
|---------------------|---------------------|
| min                 | % positive          |
| 0                   | 7                   |
| 5–9                 | 41                  |
| 10–14               | 47                  |
| 15–19               | 45                  |
| 20–25               | 45                  |
| 60                  | 49                  |
| 120                 | 45                  |
| 240                 | 44                  |

**TABLE I**  
*Time course of Glut4 translocation following microinjection of GST-IRAP-(55–82)*

3T3-L1 adipocytes were plated on coverslips with grids and incubated in serum-free medium for 2 h. Adipocytes were microinjected with 20 mg/ml GST-IRAP-(55–82). Both the time of injection and the location of the injected adipocytes within the grid matrix were recorded during the experiment. The cells were maintained at 25 °C and then fixed with methanol such that the elapsed time between microinjection and fixation was known. Adipocytes were immunostained with anti-GST and anti-Glut4 (1F8) as described under “Experimental Procedures.” The percentage of microinjected adipocytes that had plasma membrane staining for Glut4 was determined. These data are representative of two experiments.

| Microinjected protein | Glut4 translocation |
|-----------------------|---------------------|
| % positive            |                     |
| 15 mg/ml GST          | 5                   |
| 1 mg/ml GST-IRAP-(55–82) | 24                  |
| 3 mg/ml GST-IRAP-(55–82) | 30                  |
| 5 mg/ml GST-IRAP-(55–82) | 34                  |
| 10 mg/ml GST-IRAP-(55–82) | 51                  |
| 20 mg/ml GST-IRAP-(55–82) | 54                  |

**TABLE II**  
*Effect of the GST-IRAP-(55–82) concentration on Glut4 translocation*

3T3-L1 adipocytes were plated on coverslips and incubated in serum-free medium for 2 h. Cells were microinjected with the indicated concentrations of GST or GST-IRAP-(55–82) fusion protein. The percentage of microinjected adipocytes that had plasma membrane staining for Glut4 was determined. These data are representative of two experiments.
In this paper we have demonstrated that microinjection of the amino terminus of IRAP causes translocation of Glut4 to the cell surface of 3T3-L1 adipocytes in the absence of insulin. Since we did not inject the catalytic domain of IRAP, the redistribution of Glut4 was not the result of introducing amineopeptidase activity into the cytoplasm of 3T3-L1 cells. Instead, this effect was mediated by the intracellular portion of the protein. We propose a model whereby the cytoplasmic domain of IRAP competes for binding with an intracellular protein that is responsible for the sorting or retention of both Glut4 and IRAP within a specialized compartment. The effect on Glut4 translocation was extremely rapid, occurring within 5–10 min following injection (Table I). The rapidity of this response was roughly comparable with that following stimulation with insulin and suggests an effect of IRAP-(55–82) on Glut4 exocytosis. Analysis of kinetic constants indicates that inhibition of Glut4 internalization is unlikely to cause maximal Glut4 surface expression in this time frame (19, 23). Thus the rapid increase in cell surface Glut4 must be the result of an increase in the rate of exocytosis. Although injected IRAP-(55–82) could also affect the rate of Glut4 internalization, kinetic studies examining the individual rate constants will be needed to definitively answer this question.

Unlike insulin-stimulated Glut4 translocation, the translocation of Glut4 caused by injection of IRAP was independent of PI3K activation. Neither wortmannin pretreatment or co-injection of the n-SH2 domain from the PI3K regulatory subunit blocked the effect of IRAP. Both of these manipulations block insulin-stimulated Glut4 translocation (21, 22). In addition, injection of IRAP did not cause plasma membrane ruffling, a process that is stimulated in 3T3-L1 adipocytes by activated PI3K (18). Thus the mechanism by which IRAP stimulates Glut4 translocation appears to be distal to PI3K activation and is consistent with our hypothesis that injection of IRAP may cause translocation by saturating a retention receptor.

Deletion analysis of IRAP indicates that there are multiple regions within the amino terminus that affect the intracellular distribution of Glut4. GST fusion proteins containing residues 1–52 or 55–82 of IRAP were both active in promoting Glut4 translocation in 3T3-L1 adipocytes. It is not readily apparent why both of these regions were active in this assay. IRAP-(55–82) contains one dileucine motif (amino acids 76–77), which may have a role in the intracellular sequestration of Glut4, but IRAP-(1–52) does not. There is, however, a diverine motif (amino acids 27–28) present in the 1–52 region. Could this motif substitute for the dileucine motif present in the other effective regions of IRAP and the carboxyl-terminal tail of Glut4? Interestingly, the dileucine motif itself does not appear to be sufficient, since the dileucine motif (amino acids 53 and 54) in IRAP-(35–59) was not effective in stimulating translocation. This is consistent with experiments in 3T3-L1 adipocytes in which the dileucines functioned only as an internalization motif (10). Other residues surrounding the dileucines may be important as has been suggested previously from Glut1/Glut4 chimera studies in L6 cells (7). Both the 1–52 and 55–82 regions contain acidic residues, and it is possible that these charged residues may also be important. Alternatively, IRAP-(1–52) and IRAP-(55–82) may stimulate Glut4 exocytosis by two different mechanisms, each mediated by independent interactions.

A potential model for Glut4 recognition by the hypothesized retention receptor is the tyrosine-based motifs recognized by the adaptin complexes of AP-1 and AP-2 which mediate binding to clathrin (24). These large multisubunit protein complexes recognize tyrosines within the context of NPXY or YXXΦ,
where $X$ is any amino acid and $\phi$ is any amino acid with a bulky hydrophobic group (e.g. Phe, Leu, Ile, Val, Lys). AP-1 and AP-2 cross-link cell surface proteins containing these motifs with clathrin complexes, thereby concentrating these proteins for internalization into early endosomes. In a similar manner, the retention receptor may recognize and bind a trafficking motif within Glut4 and IRAP, which results in enrichment of these proteins within a vesicular compartment. One interesting question is how these complexes might be regulated by insulin. As PI3K has been implicated in insulin stimulation of glucose transport, the phosphoinositides generated by insulin action might interact with this complex to release Glut4 and IRAP.

We think that the data from our experiments are consistent with the following hypothesis: the amino terminus of IRAP and the carboxyl terminus of Glut4 share a common trafficking motif that interacts with an intracellular protein (retention receptor/sorting protein), resulting in sequestration of both IRAP and Glut4 within a specialized intracellular compartment. Injection of the amino terminus of IRAP competes with endogenous IRAP and Glut4 for retention receptor binding sites and results in exocytosis of Glut4. It will be very interesting to pinpoint the requirements for retention and to identify cellular proteins that interact with this region, since they may be critical in controlling intracellular trafficking of the Glut4 vesicle.

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