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Trafficking Kinetics of the Insulin-Regulated Membrane Aminopeptidase in 3T3-L1 Adipocytes

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In fat and muscle cells insulin causes the marked translocation of the glucose transporter GLUT4 from its intracellular location to the plasma membrane. We and others have discovered an insulin-regulated membrane aminopeptidase (designated IRAP) that colocalizes with intracellular GLUT4 and also translocates markedly in response to insulin. This study describes the trafficking kinetics of IRAP in 3T3-L1 adipocytes. By means of a surface biotinylation method, the half-time for the increase in IRAP at the plasma membrane in response to insulin was found to be 2 min. The increase was completely blocked by the phosphatidylinositol 3-kinase inhibitor, wortmannin. In insulin-treated cells, biotinylated IRAP, initially at the plasma membrane, equilibrated with the intracellular pool with a half-time of 2 min. Thus, IRAP continuously recycles. Finally, vesicles isolated from the intracellular membranes with antibodies against IRAP and GLUT4 showed the same protein composition. In conjunction with results in the literature, these findings indicate that IRAP and GLUT4 traffic through the same intracellular compartments. © 1997 Academic Press

Insulin stimulates the translocation of the glucose transporter isotype GLUT4 to the cell surface in fat and muscle cells (1). Most likely this is due to the increased exocytosis of specialized vesicles containing GLUT4, but it is also possible that it is due in part to release of GLUT4 retained in an endosomal compartment (2). We have recently cloned a membrane aminopeptidase that behaves similarly to GLUT4, now designated IRAP for *i*nsulin-*r*egulated *a*mino*p*eptidase (3). In rat and 3T3-L1 adipocytes, IRAP largely colocalizes with GLUT4, as shown by subcellular fractionation (4-6), by vesicle immunoadsorption with antibodies against either GLUT4 or IRAP (4-6), by immunofluorescence of the two proteins (6, 7), and by immunoelectron microscopy of the two proteins in the intracellular membrane fraction (8). Like GLUT4, IRAP is primarily located intracellularly in basal adipocytes and markedly translocates to the cell surface in response to insulin (4-6).

The kinetics of IRAP trafficking have not previously been determined. In the present study, we have examined these in 3T3-L1 adipocytes by procedures that involve tagging IRAP at the cell surface through biotinylation. Our results show that in response to insulin, IRAP very rapidly translocates to the plasma membrane, and that in the insulin-treated state it continuously and rapidly cycles from the plasma membrane through intracellular membranes. In these properties the trafficking of IRAP is quantitatively virtually the same as that of GLUT4.

MATERIALS AND METHODS

Antibodies. The antibodies against IRAP and GLUT4 used throughout were the affinity-purified rabbit antibodies against the entire intracellular domain of IRAP and the carboxyl terminal 19 amino acids of GLUT4 described previously (3, 9).

Cell culture. 3T3-L1 fibroblasts were cultured and differentiated into adipocytes as described in (10). Adipocytes were used at 8-12 days after the initiation of differentiation. Cells were places in serum-free Dulbecco's modified Eagle's medium (DMEM) for 2 h prior to use.

Quantitation of cell surface IRAP by biotinylation. Plates (35 mm) of cells at 37°C in Krebs-Ringer phosphate buffer (KRP) (10) with 1 mg/ml bovine serum albumin were treated with insulin or left in the basal state, as described in the legends of Figs. 1 and 2. Cell surface biotinylation, isolation of IRAP, and measurement of its relative biotin content by immunoblotting were performed, exactly as described in detail in (6). In this method, the cells are rapidly cooled to 4°C by three washes with cold KRP with 20 mM Hepes, pH 7.4 (KRBH), in order to stop membrane trafficking and then reacted with 1 mM sulfo-NHS-LC-biotin (Pierce), a membrane-impermeant reagent, for 30 min in this buffer. The cells are then lysed in buffer containing the nonionic detergent nonylethyleneglycol dodecyl ether ($C_{12}E_9$), and over 90% of the IRAP is immunoadsorbed from the clarified lysate. The relative amounts of biotin linked to the isolated IRAP is determined by immunoblotting with streptavidin-horseradish peroxidase, and the relative amounts of IRAP itself is determined by reprobing the blot with anti-IRAP.

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Internalization of IRAP. Plates (35 mm) of cells were treated with 170 nM insulin in DMEM for 15 min at 37°C, cooled to 4°C by washing with KRBH at 4°C, and reacted with sulfo-NHS-LC-biotin as described above. The plates were washed twice with KRBH at 4°C to remove unreacted biotinylating reagent. Then each plate was rapidly brought to 37°C by putting it in a 37°C water bath and adding 1 ml of KRP with 1 mg/ml bovine serum albumin and 170 nM insulin. At various times thereafter, over a 30 second period, each plate was rapidly washed twice with 20 mM Hepes/1 mM EDTA/225 mM sucrose, pH 7.4 (HES) at room temperature and homogenized in 1 ml HES with protease inhibitors (1 μM phenylmethanesulfonyl fluoride, 10 μM leupeptin, 10 μM EP475, 1 μM pepstatin A, 10 μg/ml aprotinin) by 10 strokes of a rotating teflon pestle (1200 rpm) in a glass homogenizer tube. The homogenate was centrifuged at 48,000 x g_{max} for 15 min. The supernatant, which contains the low density microsomes (LDM) and cytosol (11), was made 100 mM in NaCl and 0.5% in $C_{12}E_9$, to solubilize the LDM, and then IRAP was immunoprecipitated and its relative biotin content measured by blotting with streptavidin, as described above.

Isolation of IRAP and GLUT4 vesicles. This procedure is a slight modification of that previously described for rat adipocytes (4). Plates (10 cm) of 3T3-L1 adipocytes were washed with HES and homogenized in 3 ml of HES with protease inhibitors, as described above. A series of centrifugations (16,000 x g_{max} for 15 min, 48,000 x g_{max} for 15 min, 180,000 x g_{max} for 1.5 h) yielded the LDM as a pellet, which was then homogenized in HES/100 mM NaCl (2 ml for the LDM derived from one plate) with protease inhibitors. The suspension was first precleared by adsorption with irrelevant rabbit IgG bound to protein A on the surface of formaldehyde-fixed Staph A cells (11 μg on 4 μl Staph A cells) for 2 h at 4°C. The preclearing adsorbent was removed by centrifugation, and then IRAP or GLUT4 vesicles were absorbed with affinity-purified antibodies against the cytoplasmic domain of IRAP or the carboxy terminus of GLUT4, respectively, attached to the Staph A adsorbent (11 μg antibodies on 4 μl Staph A cells) for 2 h at 4°C. A control adsorption was carried out with irrelevant rabbit IgG. The adsorbates were pelleted and washed three times with HES/100 mM NaCl. The vesicles were solubilized in 0.5% $C_{12}E_9$ in HES/100 mM NaCl with protease inhibitors (75 μl); and the proteins released from the adsorbent were separated by sodium dodecylsulfate gel electrophoresis, transferred to nitrocellulose, and stained for protein with colloidal gold (BioRad).

RESULTS AND DISCUSSION

Time course of the insulin-elicited translocation of IRAP to the cell surface. We have previously developed a method to measure the relative amounts of IRAP at the surface of 3T3-L1 adipocytes by biotinylation with a membrane-impermeant reagent and shown that insulin treatment for 15 min results in an approximately 8-fold increase in IRAP at the cell surface (6). In the present study, this method has been used to determine the time course for the translocation of IRAP. The data in Fig. 1 show that the half-time for IRAP translocation after insulin addition was approximately 2 min. The maximal level of IRAP at the cell surface, which was reached at approximately 5 min, was then constant for a period of at least 90 min.

An extensive kinetic modeling of the trafficking of GLUT4 in adipocytes has led to the conclusion that the observed half-time for the appearance of GLUT4 at the cell surface upon insulin addition is substantially determined by the rate constant for exocytosis of GLUT4 vesicles (12). Since IRAP and GLUT4 are colocalized in these vesicles, their half-time for translocation would be expected to be the same. In fact, they are. The half-time for appearance of GLUT4 at the surface of 3T3-L1 adipocytes, measured by photoaffinity labeling of surface GLUT4, is also 2 min (12).

Effect of wortmannin on insulin-elicited IRAP translocation. Pretreatment of 3T3-L1 adipocytes with the phosphatidylinositol 3-kinase inhibitor, wortmannin, at 100 nM concentration, completely blocks insulin-elicited GLUT4 translocation (15). In order to determine whether the same is true for IRAP, the change in IRAP at the cell surface in response to insulin was measured in the absence or presence of wortmannin by the cell surface biotinylation method. The results in Fig. 2 show that in the absence of wortmannin, the biotin content of IRAP from insulin-treated cells showed the expected increase (here about 6-fold) over that from basal cells, whereas in the presence of wortmannin there was no increase. Thus, wortmannin completely blocked IRAP translocation. Previously we have

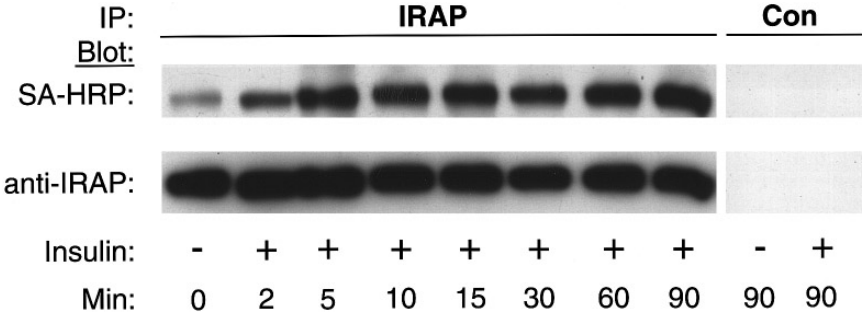

FIG. 1. Time course of IRAP translocation in response to insulin. Plates (35 mm) of 3T3-L1 adipocytes were either in the basal state or treated with 170 nM insulin at 37°C. At the stated times after addition of insulin, the cells were cooled to 4°C to prevent further membrane trafficking, and then surface biotinylated with sulfo-NHS-LC-biotin. Samples of the cell lysates were immunoprecipitated with antibodies against IRAP or irrelevant antibodies (Con). Biotinylation of IRAP was detected by blotting with streptavidin conjugated to horseradish peroxidase (upper panel). Each lane contained IRAP immunoadsorbate derived from 25% of a 35 mm plate. In order to verify that the loadings were equivalent, the relative amounts of immunoprecipitated IRAP were determined by reprobing the membrane with antibodies against IRAP (lower panel). Similar results were obtained in two repetitions of this experiment.

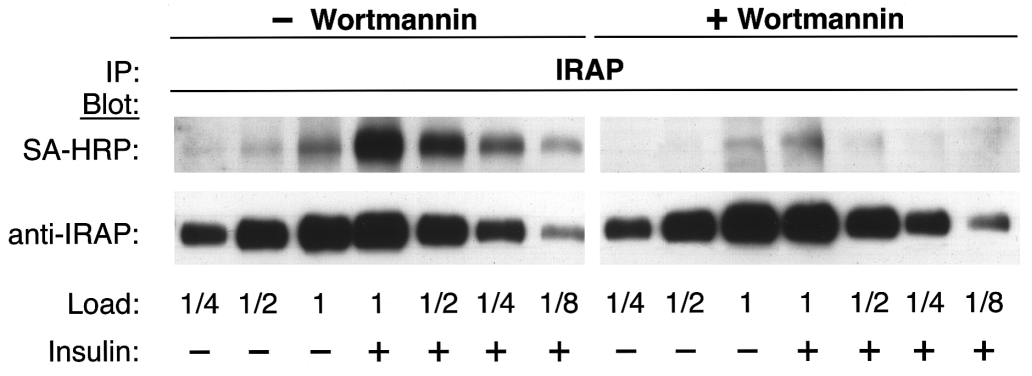

FIG. 2. Inhibition of insulin-elicited IRAP translocation by wortmannin. Plates (35 mm) of 3T3-L1 adipocytes were incubated at 37°C with or without 100 nM wortmannin for 10 min, then also treated with 170 nM insulin for 15 min (+) or not (−), and then surface biotinylated with sulfo-NHS-LC-biotin at 4°C. Samples of the cell lysate were immunoprecipitated with antibodies against IRAP. Biotinylation was detected by blotting with streptavidin conjugated to horseradish peroxidase (upper panel). The relative amounts of immunoprecipitated IRAP were determined by reprobing the membrane with antibodies against IRAP (lower panel). The loads of IRAP immunoadsorbate per lane were as shown, where load *1* was derived from 10% of a 35 mm plate of adipocytes. A repetition of this experiment yielded identical results.

shown that the insulin-elicited translocation of IRAP in 3T3-L1 adipocytes is accompanied by an increase in the aminopeptidase activity of the intact cells toward vasopressin in the medium (16). In agreement with the effect of wortmannin on IRAP translocation, pretreatment of 3T3-L1 adipocytes with 100 nM wortmannin also completely blocked the insulin elicited increase in this cell surface aminopeptidase activity (data not shown).

There is now strong evidence that the activation of phosphatidylinositol 3-kinase is a step in the signaling pathway from the insulin receptor to GLUT4 translocation (summarized in (17)). The inhibitory effect of wortmannin on IRAP translocation indicates that the same signaling pathway causes its translocation.

Recycling of IRAP. In order to determine whether IRAP is continuously being internalized and returned to the cell surface, we measured the intracellular appearance of biotinylated IRAP that was initially entirely at the cell surface. Plates of insulin-treated 3T3-L1 adipocytes were surface biotinylated at 4°C, incubated at 37°C for various periods in the presence of insulin, and then homogenized. The LDM fraction, in which intracellular vesicles containing the IRAP are located (6), was prepared. The IRAP in this fraction was isolated by immunoadsorption and its biotin content was assayed by immunoblotting. The results of a representative experiment are presented in Fig. 3. The biotin content of the IRAP in the LDM rose from a low level to a much higher steady-state level over a period of 5 min, with a half-time of approximately 2 min. The increase in the biotin content of IRAP in the LDM was not the result of a net internalization of IRAP, since the IRAP content of the LDM remained constant between 0

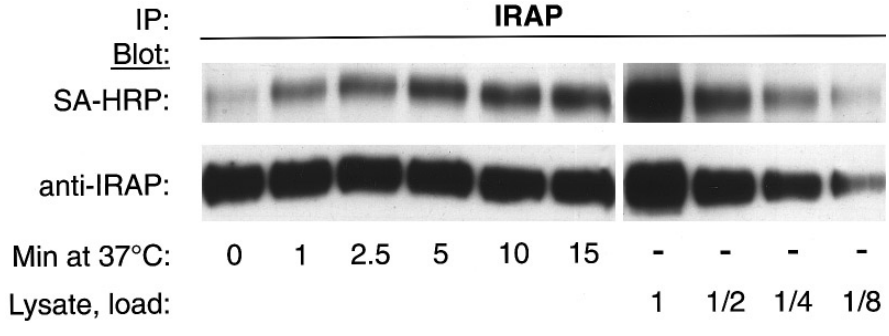

FIG. 3. Time course of IRAP recycling in insulin-treated 3T3-L1 adipocytes. Plates (35 mm) of 3T3-L1 adipocytes, previously treated with 170 nM insulin for 15 min at 37°C, were surface biotinylated with sulfo-NHS-LC-biotin at 4°C. The cells were then warmed to 37°C and incubated for the indicated times in the presence of 170 nM insulin before homogenization. The LDM fraction was prepared by differential centrifugation, and IRAP was immunoprecipitated therefrom. Biotinylated IRAP was detected by blotting with streptavidin conjugated to horseradish peroxidase (upper panel). The relative amounts of immunoprecipitated IRAP were determined by reprobing the membrane with antibodies against IRAP (lower panel). Each lane contained the amount of IRAP derived from the LDM from 20% of a 35 mm plate. The four right-hand lanes show IRAP obtained by immunoprecipitation from a total lysate of biotinylated, insulin-treated cells not warmed to 37°C (load *1* derived from 10% of a 35 mm plate). Similar results were obtained in two repetitions of this experiment.

and 15 min (Fig. 3, lower panel). Thus, as biotin-labeled IRAP at the cell surface was internalized, unlabeled intracellular IRAP trafficked to the cell surface, such that the steady-state subcellular distribution of IRAP protein did not change.

Figure 3 also includes data for IRAP isolated from a total cell lysate (right lanes). The IRAP signal given by IRAP isolated from 5% of a plate (lane with 1/2 load) is approximately the same as that given by the IRAP isolated from the LDM of 20% of a plate (0-15 min). Thus, approximately 25% of the IRAP was recovered in the LDM fraction. After the steady-state distribution of the biotin-labeled IRAP was achieved, the specific biotin content of the IRAP in the LDM, defined as the ratio of biotin signal to IRAP signal, was the same as that for the total cellular IRAP (compare 5-15 min and 1/2 load in Fig. 3). This result indicates that all or most of the IRAP in the LDM vesicles equilibrated with the biotin-labeled IRAP initially at the cell surface.

These results are similar to those obtained for the recycling of photoaffinity-labeled GLUT4 in insulin-treated 3T3-L1 adipocytes (13). In the case of GLUT4, the half-time for redistribution of the labeled transporter is 4 min, a value similar to but somewhat larger than the value of approximately 2 min for IRAP. It is unclear whether this difference is significant, since the measurements involved different techniques with different lines of 3T3-L1 cells. However, in principle, the half-times for the steady-state recycling of IRAP and GLUT4 could differ. This half-time depends in part upon the rate constant for internalization of the protein, as described in (12, 13). The rate constant for internalization of GLUT4 has been shown to be determined by a dileucine motif in its carboxy terminal cytoplasmic domain (14), and IRAP has two such motifs in its cytoplasmic domain, one or both of which are likely to have the same function (3). Since the amino acid sequences surrounding these dileucines are not the same in GLUT4 and IRAP, there is no reason to expect that their rate constants for internalization should be identical.

IRAP and GLUT4 vesicles. The intracellular vesicles containing IRAP and GLUT4 were isolated from the LDM fraction by immunoadsorption with antibodies against each protein bound to protein A on formaldehyde-fixed Staph A cells. We have previously shown that vesicles isolated in this way with anti-IRAP (designated IRAP vesicles) contain over 90% of the GLUT4, and similarly, vesicles isolated with anti-GLUT4 (designated GLUT4 vesicles) contain over 90% of the IRAP, whereas an irrelevant IgG adsorbs vesicles containing neither IRAP or GLUT4 (6). Here we have examined the protein compositions of the vesicles (Fig. 4). The preparations of IRAP and GLUT4 vesicles show a number of bands of the same mobility and equal intensity as those in the adsorbate with irrelevant IgG (lane C).

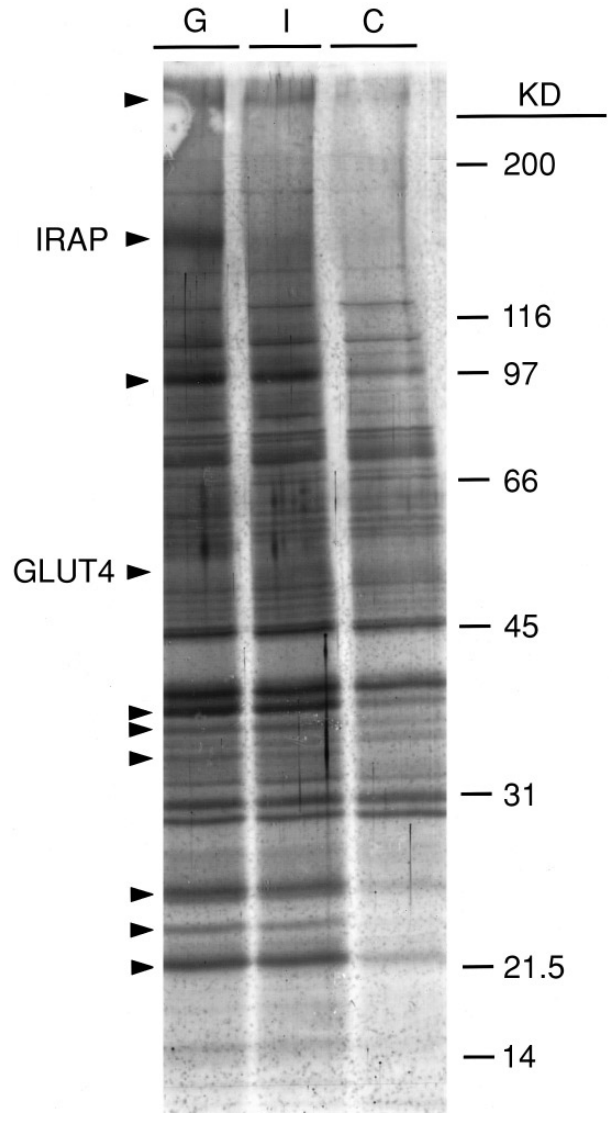

FIG. 4. Protein compositions of IRAP and GLUT4 vesicles. Vesicles were adsorbed from the LDM fraction of 3T3-L1 adipocytes with antibodies against GLUT4 (G) or IRAP (I), or with irrelevant rabbit IgG (C) bound to Staph A cells. Adsorbed vesicles were solubilized with $C_{12}E_9$; the released proteins were resolved on an 8-16% gradient gel, transferred to nitrocellulose, and stained with colloidal gold. Each lane contains the vesicle proteins derived from one 10 cm plate of 3T3-L1 adipocytes. Arrowheads on the left designate bands specific to the IRAP and GLUT4 vesicles. This result is representative of three similar experiments.

These are presumably nonspecifically adsorbed proteins. In addition, there are a number of bands either only present or much more intense in the IRAP and GLUT4 vesicles, which are denoted by arrowheads on left of Fig. 4. These are presumably proteins specific to or enriched in the IRAP and GLUT4 vesicles. A comparison of the two types of vesicles shows that they are the same in the composition and amount of these specific proteins. Also noted on Fig. 4 is the IRAP band, which

is seen only in the GLUT4 vesicles (lane G), and a faint band designated GLUT4 because of its correct size (50 kD) and shape (broadish), which is seen only in the IRAP vesicles (lane I). The absence of IRAP in the mixture of IRAP vesicle proteins and of GLUT4 in the mixture of GLUT4 vesicle proteins is due to the fact that when the IRAP (or GLUT4) vesicles are solubilized with $C_{12}E_9$, the IRAP (or GLUT4) remains bound to its antibodies on the Staph A adsorbent (6).

The identical protein compositions of the IRAP and GLUT4 vesicles provides further evidence that these two proteins are entirely colocalized in 3T3-L1 adipocytes and thus traffic through the same intracellular compartments.

Conclusion. These results provide further support for the conclusion that in adipocytes IRAP and GLUT4 exhibit highly similar, if not identical, trafficking, including the regulation by insulin. Recent studies in this area have led to the conclusion that the cytoplasmic carboxy terminal domain of GLUT4 contains a motif that directs the intracellular retention of GLUT4 in unstimulated cells and thus is required for the marked translocation in response to insulin (14, 18, 19), and that this motif is distinct from the dileucine motif that controls the rate of endocytosis (14, 18). The similar trafficking of IRAP and GLUT4 is evidence that the same motif also occurs in the 109 amino acid cytoplasmic domain of IRAP. Current efforts are focused on the identification of this retention motif. The existence of two proteins with this motif will facilitate its definitive identification.

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