Insulin-responsive Aminopeptidase Trafficking in 3T3-L1 Adipocytes

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The insulin-responsive aminopeptidase (IRAP/VP165/gp160) was identified originally in GLUT4-containing vesicles and shown to translocate in response to insulin, much like the glucose transporter 4 (GLUT4). This study characterizes the trafficking and kinetics of IRAP in exocytosis, endocytosis, and recycling to the membrane in 3T3-L1 adipocytes. After exposure of 3T3-L1 adipocytes to insulin, IRAP translocated to the plasma membrane as assessed by either cell fractionation, surface biontinylation, or the plasma membrane sheet assay. The rate of exocytosis closely paralleled that of GLUT4. In the continuous presence of insulin, IRAP was endocytosed with a half-time of about 3–5 min. IRAP endocytosis is inhibited by cytosol acidification, a property of clathrin-mediated endocytosis, but not by the expression of a constitutively active Akt/PKB. Arrival in an LDM fraction derived via subcellular fractionation exhibited a slower time course than disappearance from the cell surface, suggesting additional endocytic intermediates. As assayed by membrane “sheets,” GLUT4 and IRAP showed similar internalization rates that are wortmannin-insensitive and occur with a half-time of roughly 5 min. IRAP remaining on the cell surface 10 min following insulin removal was both biotin- and avidin-accessible, implying the absence of thin-necked invaginations. Finally, endocytosed IRAP quickly recycled back to the plasma membrane in a wortmannin-sensitive process. These results demonstrate rapid endocytosis and recycling of IRAP in the presence of insulin and trafficking that matches GLUT4 in rate.

The metabolic hormone insulin promotes the disposal of glucose into its peripheral target tissues, adipose and muscle. Insulin-stimulated glucose uptake is mediated primarily by the rapid movement of the fat/muscle-specific glucose transporter (GLUT)1 from a latent intracellular compartment to the cell surface (1). The subcellular trafficking of GLUT4 has been studied using an impermeant photolabel specific for the extra-cellular sugar-binding site of the transporter (2–4). These studies demonstrated that in the presence of insulin there was constant recycling of GLUT4 between the intracellular compartment and the plasma membrane, although the total amount of GLUT4 in the plasma membrane was elevated and constant. There are data supporting the existence of exocytotic intermediates in which GLUT4 might be present in the membrane but inaccessible to substrate (2). Also, a recent report studying GLUT4 in CHO cells demonstrated endocytic intermediates similar to those described for a synaptic vesicle protein, synaptophysin (5, 6).

Recently, the insulin-responsive aminopeptidase (IRAP) has been cloned, shown to reside in the GLUT4-containing basal compartment, and also translocate to the plasma membrane after insulin stimulation (7, 8). The physiological function of IRAP has been hypothesized to be hormonal modulation of circulating vasoactive peptides such as vasopressin (9, 10). Indeed, determining the normal function of IRAP might be critical in defining the mechanism of diabetic complications such as hypertension or peripheral vascular disease that might result from impaired translocation of IRAP. Therefore, investigating the normal trafficking and biology of IRAP is critical not only to illuminate insulin-stimulated trafficking but as a first step in eventually defining pathologies in conditions such as diabetes.

The purpose of this study was to describe the trafficking and kinetics of IRAP in the context of GLUT4 in 3T3-L1 adipocytes. Several studies have shown overlapping subcellular distribution of GLUT4 by light microscopy (11, 12) and subcellular fraction (13, 14). Whereas the steady state location of IRAP is overlapping with GLUT4, its trafficking has not been described in detail. One report suggests that IRAP and GLUT4 traffic differently in response to high glucose or glucosamine (15). Another demonstrates IRAP exclusion from cardiac secretory granules containing atrial natriuretic factor and GLUT4 (11). Finally, a third report studying rat fat demonstrates that IRAP does not internalize in the presence of insulin (16); this is in marked contrast to the constitutive recycling of GLUT4.

In an effort to define the trafficking and kinetics of IRAP movement, we have adapted protocols using biontinylation to develop assays measuring endocytosis and recycling of IRAP back to the cell surface. Additionally, we employed the plasma membrane sheet protocol to compare directly IRAP and GLUT4 levels on the cell surface. By using these techniques we find IRAP is endocytosed rapidly both in the presence and absence of insulin. Moreover, IRAP is endocytosed and exocytosed with rates similar to GLUT4. These techniques have also allowed us to study other details of IRAP as follows: its endocytosis is inhibited by cytosolic acidification, a property of clathrin-mediated endocytosis, but not by a constitutively active Akt/PKB. Endocytic intermediates likely occur between exiting the

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plasma membrane and arrival in the LDM. Finally, we can directly demonstrate endocytosed IRAP rapidly recycles to the plasma membrane in the presence of insulin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Crystalline porcine insulin was a gift of Lilly. Polyclonal antibodies against IRAP cryoplastic amino terminus were kindly donated by Metabolex, Inc. (Hayward, CA). Polyclonal sheep anti-GLUT4 antibodies were raised against a glutathione S-transferase fusion protein encoding the last 31 amino acids of the GLUT4 carboxyl terminus. Monoclonal mouse anti-IRAP were raised against the 110 amino acids of the IRAP amino terminus. Affinity purified/subtracted rhodamine-conjugated donkey anti-sheep and affinity purified/subtracted fluorescein isothiocyanate-conjugated donkey anti-mouse antibodies were purchased from Jackson ImmunoResearch (West Grove, Pa.). Bovine serum albumin used in transfection assays was from Calbiochem. Wortmannin was purchased from Sigma and stored as a 10 mM stock in MeSO. 125I-Protein A was purchased from ICN Radiochemicals (Irvine, CA). A bicinchoninic acid protein assay kit for the determination of protein concentrations was from Pierce as were 2,2'-azino-di(3-ethylbenzithiazoline sulfonate), streptavidin-HRP, neutravidin-HRP, sulfo-NHS-LC-biotin, and sulfo-NHS-S-s-biotin. Unless otherwise noted, all other chemicals were from Sigma.

**Cell Culture—**3T3-L1 fibroblasts were grown at 37 °C in a humidified atmosphere of 7.5% CO2 in Dulbecco's modified Eagle's medium containing 10% calf serum (Life Technologies Inc.). Cells were plated onto either 18-mm square number 1 coverslips, 6-well or 10-cm plates, containing 10% calf serum (Life Technologies Inc.). Cells were plated at a density of 100,000 cells/cm2 and differentiated 2 days postconfluence with dexamethasone (0.4 mg/mL) and insulin (10 nM) to induce adipogenesis. 3T3-L1 adipocytes were resuspended in HES containing 10% calf serum, and incubated with neomycin (1 mg/mL) for 3 days, and then differentiated 2 days postconfluence with dexamethasone (0.4 mg/mL) and insulin (10 nM) to induce adipogenesis. Cells were washed twice in ice-cold KRPH (128 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl2, 1.25 mM MgSO4, 5 mM NaPO4, 20 mM Hepes, pH 7.4) and treated with 1 mM of 0.5 mM sulfo-NHS-LC-biotin in KRPH for 30 min. Each plate was then bathed three times for 10 min each in KRPH containing 20 mM glycine, twice with KRPH, and finally lysed in 1 mL of Solubilization Buffer (1% Triton, 150 mM NaCl, 20 mM Tris-Cl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM/ml aprotinin, 10 μM leupeptin, 1 μM pepstatin A, pH 7.4). The lysate was vortexed briefly, incubated for 15 min, and centrifuged at 23,000 × g for 15 min. After passing the supernatants through a 45-μm filter, the supernatant was subjected to differential centrifugation. The supernatant from the following spins were serially removed and pelleted in a Ti70 rotor as follows: 19,000 × g (20 min), 41,000 × g (20 min), and 180,000 × g (75 min). The first 19,000 × g pellet was resuspended, loaded onto a sucrose cushion (1.12 M sucrose, 20 mM Hepes, pH 7.4, 1 mM EDTA), and isolated from the interface yielding the Plasma Membrane (PM) fraction as the pellet of a 41,000 × g spin (20 min). The last 180,000 × g pellet corresponded to the Low Density Microsome (LDM) fraction. After resuspension of pellets in Solubilization Buffer, protein concentrations were determined by the bicinchoninic acid protein assay. Reducing agent in SDS-PAGE sample buffer. Alternatively, cell lysates were incubated with streptavidin-agarose for 1 h and eluted with sam- ple buffer containing 1 mg/mL streptavidin-HRP. Data are expressed either as “Cell Surface IRAP” or “Percent Escaping Cleavage.” The former is determined by first subtracting the value of biotinylation after immediate cleavage from all other values, dividing by total uncleaved signal, and subtracting from 1. The latter is determined by first subtracting the value of biotinylation after immediate cleavage from all other values and dividing by maximum protected signal.

**Cytosolic Acidification**—As described previously (26, 27), cells were exposed to a media containing acetic acid in order to acidify cytosol. After biotinylation and glycine treatment cells were washed twice in Leibovitz-15, pH 5.0 (HCl), washed twice in Leibovitz-15, pH 5.0 (acetic acid), and left in a third wash of Leibovitz-15, pH 5.0 (acetic acid), for 30 min at 4 °C. Cells were exposed to identical media prewarmed to 37 °C,
RESULTS

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Adipocytes, we measured insulin-stimulated translocation to the cell surface by three complimentary methods. The first was a quantitative biotinylation protocol adapted from published studies (8, 14). Insulin treatment induced a large increase in IRAP available at the cell surface for biotinylation (Fig. 1A). We next applied techniques that are useful for measuring GLUT4 translocation as well as that of IRAP. After stimulating cells with insulin or maintaining them in a basal state, we homogenized cells and fractionated them into Plasma Membrane (PM), and Low Density Microsome (LDM) fractions (Fig. 1B) (30). Insulin elicited a dramatic redistribution of IRAP from the LDM fraction to the PM fraction, similar to GLUT4 (31). Finally, to compare directly trafficking of GLUT4 and IRAP in the same cells, we labeled membrane sheets for the presence of IRAP and GLUT4 (Fig. 1C). Quantitation of images revealed a similar degree of translocation between the two proteins. These results demonstrating insulin-stimulated translocation of IRAP are consistent with previous reports (12, 14, 32) and serve to emphasize the parallel between IRAP and GLUT4 trafficking.

In the Presence of Insulin IRAP Is Rapidly Endocytosed and Continues to Recycle to the Plasma Membrane

A recent report demonstrated that IRAP does not endocytose in the presence of insulin in rat fat (16). To measure internalization in our cells, we adapted a reversible biotinylation technique to pulse-chase labeled IRAP into cells and determine the amount remaining on the surface by its susceptibility to a membrane-impermeable cleavage reagent. Cells were stimulated with insulin, biotiny-
IRAP were serum-deprived for 2 h in Leibovitz-15 medium containing 0.2% BSA and stimulated with or without 20 nM insulin for the final 20 min. Cells were chilled at 4 °C to label IRAP, and rewarmed in the presence of insulin with a 1/2 of approximately 3 min.

To determine the kinetics of the entire population of IRAP, cells were biotinylated at 37 °C in the presence of insulin for increasing times, and afterward biotinylated material was precipitated from cell lysates with avidin-agarose as in Fig. 2A. Precipitated material was then assayed for IRAP by Western blot analysis and compared with total cellular IRAP. Fig. 2C demonstrates a time-dependent increase in IRAP biotinylation such that virtually all IRAP in the cell has translocated to the cell surface at least once by 1 h. Furthermore, since cell surface levels of IRAP are constant after 10 min of insulin stimulation (Fig. 5A), these data indicate that intracellular stores of IRAP are constantly driven to the cell surface to replace the internalized IRAP demonstrated in Fig. 2, A and B.

**IRAP Endocytosis Is Inhibited by Cytosol Acidification but Not by myr-Akt**—Cytosolic acidification has been shown to inhibit potentially clathrin-mediated endocytosis by disruption of the clathrin lattice (33). Insulin-treated, biotinylated cells were warmed for 2 or 5 min in regular or cytosolic acidification media and then exposed to cleavage buffer (Fig. 3A). For the final washout lane, cells were warmed for 5 min in acidification media, chilled, washed, and warmed for the final 15 min in pH 7.4 media. Cytosolic acidification prevented any sequestration from cleavage buffer, but its removal allowed biotinylated IRAP to endocytose and escape cleavage. These data support a role for clathrin in the endocytosis of IRAP.

We next tested the mechanism of Akt-induced increases of plasma membrane IRAP. A constitutively active myristoylated Akt results in a large increase in IRAP and GLUT4 present at the plasma membrane (Fig. 3B, inset) (21). To test whether Akt might be inhibiting endocytosis, cells expressing a constitutively active myr-Akt construct were compared with empty vector controls in a reversible biotinylation endocytosis assay as in Fig. 2B. Both cells demonstrated similar rates indicating that inhibition of endocytosis does not account for the ability of myr-Akt to stimulate translocation of IRAP to the cell surface.

**IRAP Arrival in the LDM Is Slower Than Escape from Cleavage**—Because of the likelihood that development of biotinylated IRAP’s resistance to cleavage is a very early event in its endocytosis, the rate of escape from cleavage was compared with the rate of arrival into LDM. Sensitivity to cleavage by impermanent reducing agent was determined from experiments performed as in Fig. 2B, with the data expressed as percent escaping cleavage. Arrival into the LDM was determined by fractionating cells treated as in Fig. 2, A and B, immunoprecipitating IRAP from the LDM pellet, and assaying for biotinylated IRAP with streptavidin-HRP. At 5 min after warming there was a smaller quantity of IRAP present in LDM compared with that which is inaccessible to extracellular cleavage (Fig. 4A). To verify this delay, the escape from cleavage was determined in the same cells used to measure arrival in LDM. As shown in Fig. 4B, after 1 min of internalization, there was a statistically significant difference in the percent escaping cleavage (40%) versus that arriving in LDM (15%). This discrepancy does not simply reflect contamination of PM with LDM as indicated by its disappearance at later time points. Thus, after a 40-min chase, 60% more of the internalized IRAP is pelleted in the LDM fraction compared with the recovery of internalized IRAP in the LDM after a 1-min chase.

**IRAP Exocytic and Endocytic Rates Are Similar to GLUT4 in the Presence and Absence of Insulin, Respectively**—To compare IRAP and GLUT4 trafficking directly, plasma membrane sheets were used to assay the quantity of these proteins on the same cell surfaces at different time points after the addition or
removal of insulin. The data represented in Fig. 5A demonstrate cells treated with 20 nM insulin at time 0 and warmed for lengthening periods before surface levels of IRAP and GLUT4 were measured. IRAP and GLUT4 exocytotic rates are very similar, with a half-time of approximately 5 min. To measure endocytotic rate in the absence of insulin, a similar analysis was performed as shown in Fig. 5B. Insulin was withdrawn, and surface levels of IRAP and GLUT4 were measured after lengthening times. Again, rates of endocytosis correlate closely, with a half-time of approximately 5 min. Finally, we compared the effect of wortmannin on IRAP and GLUT4 trafficking. Wortmannin potently inhibited translocation of the two proteins if added before insulin treatment (Fig. 5C), similar to previous reports (12). When wortmannin was added after insulin stimulation in parallel to insulin withdrawal, the surface levels of IRAP and GLUT4 decreased identically. Moreover, the rates of internalization were identical to those observed when insulin was removed in the absence of wortmannin (Fig. 5C).

Plasma Membrane IRAP Is Biotin- and Avidin-accessible in the Presence and after the Withdrawal of Insulin—Recent reports (6, 34) have indicated the presence of synaptic vesicle proteins in compartments continuous with the plasma membrane but restricted by thin-necked invaginations limiting accessibility to the large (69 kDa) probe avidin. A recent report (5) has extended these findings to CHO cells exogenously expressing GLUT4 showing that GLUT4, but not transferrin receptor, has extended these findings to CHO cells exogenously expressing GLUT4 showing that GLUT4, but not transferrin receptor, was selectively sequestered in a compartment near the plasma membrane as determined by immunofluorescence. We therefore sought evidence for the presence of IRAP in such a compartment in 3T3-L1 adipocytes. To test IRAP accessibility to biotin, cells were exposed to insulin and then withdrawn from the hormone for increasing times before being chilled and biotinylated to determine the amount of accessible IRAP. Shown in Fig. 6A is a comparison of the amount of cell surface IRAP as determined by biotin accessibility and plasma membrane sheets. Given the close correlation, it is likely that the immunostained IRAP on sheets is all accessible to biotin. To determine avidin accessibility, cells either in the presence of insulin or 10 min after removal were biotinylated as above, maintained at 4 °C, and then exposed to avidin to complex all available biotin sites. The remaining avidin was quenched with biocytin, and IRAP was immunoprecipitated and Western-blotted to detect free biotin on IRAP. When 3T3-L1 adipocytes were exposed to avidin immediately after biotinylation, greater than 90% of the biotin sites on the protein were exofacially disposed and available for binding to avidin. Similar data were obtained after allowing the cells to internalize biotinylated IRAP for 10 min. Thus, IRAP appears accessible to both biotin and avidin at all time points tested.

Rapid Wortmannin-sensitive Recycling of IRAP Back to the Cell Surface—To ascertain whether there is continuous recycling of IRAP in the presence of insulin, IRAP was biotinylated, allowed to internalize for 5 min, chilled, and all remaining biotin sites on the cell surface were quenched with avidin. Cells were then rewarmed for the indicated times to allow exocytosis of internalized label. Finally, cells were chilled and exposed to neutravidin-HRP to bind newly exposed biotin residues. IRAP was immunoprecipitated and assayed for HRP activity. Fig. 7A demonstrates a time-dependent increase in HRP activity associated with IRAP. After 3 min of warm-up, 60% of maximum measured levels were achieved. In Fig. 7B, cells were exposed to 500 nM wortmannin during the final warm-up, demonstrating wortmannin sensitivity of the reappearance process.

DISCUSSION

Originally identified as a constituent of GLUT4-containing vesicles, the physiological role and trafficking pathways of IRAP still remain undefined. The current study represents an attempt to define the trafficking and kinetics of IRAP. We demonstrate four novel findings. First, IRAP trafficking resembles closely that of GLUT4. IRAP displays similar exocytosis and internalization rates, also undergoing constant recycling.

![Image](image-url)
between the plasma membrane and the LDM fraction in the presence of insulin. Moreover, IRAP is likely endocytosed via clathrin-coated pits, the means proposed for GLUT4 endocytosis (35–38). Second, the candidate insulin effector Akt does not inhibit IRAP endocytosis, indicating a truly insulinomimetic activity of the kinase on exocytosis. Third, endocytic intermediates likely occur between exit from the cell surface and arrival in LDM fraction. Fourth, IRAP is unlikely to be endocytosed via plasma membrane continuous compartments invoked for synaptic vesicle trafficking. These results complement previous reports demonstrating the steady-state colocalization of IRAP with GLUT4 and lend support to the idea of a family of insulin-responsive proteins that traffic similarly (11–14).

An important finding of this study is the continuous endocytosis of IRAP in the presence of insulin. By using a pulse-chase reversible biotinylation technique, we demonstrate the recycling of IRAP in the presence of insulin is rapid and robust, such that within 5 min of insulin-stimulated translocation, 80% of surface-labeled IRAP is intracellular. During this period when steady-state levels are unchanged, intracellular IRAP is mobilized to the cell surface to replace the endocytosed population. These data correlate well with the reported constant recycling of GLUT4 in the presence of insulin (2, 4), but the data disagree with a recent study in rat fat cells in which no cell surface-labeled IRAP appeared in the LDM over the course of 30 min of insulin stimulation (16). To reduce the likelihood that technical differences might account for this discrepancy, we have used two methods to detect endocytosed IRAP. First, we isolated biotinylated IRAP by avidin-agarose beads and stained blots with anti-IRAP antibodies comparing total IRAP protein to the amount escaping cleavage reagent. Second, we immunoprecipitated IRAP and stained blots with avidin-HRP to compare total biotin signal in IRAP to the biotin signal in IRAP.
escaping cleavage. Both techniques demonstrated endocytosis in the presence of insulin. Perhaps differences in 3T3-L1 adipocytes versus rat fat might account for the discrepancy. In agreement with the above report, we do also detect endocytosis of transferrin receptor in the presence of insulin.2

Although no previous reports have quantified IRAP internalization in adipocytes, GLUT4 internalization rates have been measured in the presence of insulin (4). The rate of internalization in 3T3-L1 adipocytes was ascertained using a labeled substrate analog covalently bound to the GLUT4 receptor by photoactivation. By following this kinetic pool in the presence of insulin, the authors determined a half-time for endocytosis of 4.2 min for GLUT4 exit from the plasma membrane fraction. This is very similar to the 3-min half-time measured for IRAP using techniques described above. Although the differences are probably within experimental variation, a functional measurement of escape from a membrane cleavage reagent could be different from movement between homogenized cell fractions, as used for GLUT4. We indeed detected a delay when comparing rates of escape from cleavage reagent to rates of arrival in the LDM. Within the same samples, there was more than a 2-fold difference in the amount of IRAP internalized into the whole cell lysate versus that recovered in the LDM after a 1-min chase. This is reflected in a significantly lower recovery of endocytosed signal in the LDM at early time points compared with ones at equilibrium; 60% more of the endocytosed signal was recovered in LDM after a 40-min chase compared with a 1-min chase. The absence of endocytosed signal from the LDM at early time points might indicate that the protected IRAP is either in a closed but pre-fission state or perhaps in an immature vesicle of sedimentation properties different than those of LDM.

When measured in parallel, GLUT4 and IRAP showed almost identical exocytic and endocytic rates, prompting the question of whether IRAP is endocytosed by a similar mechanism as GLUT4. GLUT4 has been shown by electron microscopy (39) and cytosolic acidification (40) to bud from clathrin-coated pits. IRAP possesses similar intracellular dileucine motifs to GLUT4, but these were not necessary for endocytosis of IRAP in chimerae with transferrin receptor expressed in CHO cells (41). Therefore, we assayed IRAP endocytosis under conditions inhibiting clathrin lattice formation, and we found that cytosolic acidification completely and reversibly ablated internalization, supporting a role for clathrin-mediated endocytosis of IRAP. Considering these similarities in mechanism and rate, it seems likely IRAP and GLUT4 are endocytosed by similar means.

A recent report concluded that GLUT4 endocytosis in CHO cells (5) exhibits characteristics similar to those proposed for synaptic vesicles (6, 34). These latter studies suggest that synaptic vesicle biogenesis might occur from compartments continuous with the plasma membrane but with thin-necked constrictions such that whereas biotin may access this

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2 L. A. Garza and M. J. Birnbaum, unpublished observations.
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compartment, avidin may not (6). Since IRAP and GLUT4 show similar means of endocytosis, it seemed possible that the insulin-responsive vesicle might also bud directly from the plasma membrane, particularly in light of the recent demonstration of the presence of GLUT4 in peripheral structures in CHO cells (5). However, we found that IRAP was both biotin- and avidin-accessible in the presence of insulin and, after its withdrawal, inconsistent with the hypothesis of a plasma membrane-continuous donor compartment as formulated in PC12 cells (6). A second prediction of the work in synaptic vesicles and also reported for GLUT4 in CHO cells is that cooling of cells to 15–18 °C would selectively inhibit endocytosis of IRAP but not transferrin receptor. Attempts to demonstrate this phenomenon for IRAP in 3T3-L1 cells have also been unsuccessful.2

Developing an assay for IRAP endocytosis has allowed us to ask whether the putative downstream signaling molecule from the insulin receptor Akt is stimulating exocytosis or inhibiting endocytosis. A constitutively active construct of the Akt kinase did not inhibit endocytosis of IRAP indicating that Akt increases cell surface levels of IRAP by a truly insulinomimetic capacity of stimulating exocytosis.

In conclusion, this study provides evidence for similar if not identical trafficking in rate and path between IRAP and GLUT4. The endocytosis of IRAP appears be clathrin-mediated and not analogous to models proposed for synaptic vesicle recycling. We also provide novel evidence for endocytic intermediates and the role of Akt in stimulating exocytosis. The correlation in trafficking between GLUT4 and IRAP imply that investigations of the mechanisms of GLUT4 retention and recycling are equally pertinent to IRAP. Indeed, given the capacity of microinjected IRAP amino-terminal fragments to stimulate GLUT4 translocation (42), the molecular machinery responsible for sorting these molecules will likely be identical. Future studies might further delineate these broadly outlined steps of endocytosis and recycling of IRAP into more detailed movements between specific compartments.

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