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Insulin-regulated Aminopeptidase Is a Key Regulator of GLUT4 Trafficking by Controlling the Sorting of GLUT4 from Endosomes to Specialized Insulin-regulated Vesicles

Ingrid Jordens,* Dorothee Molle,*† Wenyong Xiong,*† Susanna R. Keller,‡ and Timothy E. McGraw*

*Department of Biochemistry, Weill Medical College of Cornell University, New York, NY 10065; and
‡Division of Endocrinology, Department of Medicine, University of Virginia, Charlottesville, VA 22908

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Insulin stimulates glucose uptake by regulating translocation of the GLUT4 glucose transporter from intracellular compartments to the plasma membrane. In the absence of insulin GLUT4 is actively sequestered away from the general endosomes into GLUT4-specialized compartments, thereby controlling the amount of GLUT4 at the plasma membrane. Here, we investigated the role of the aminopeptidase IRAP in GLUT4 trafficking. In unstimulated IRAP knockdown adipocytes, plasma membrane GLUT4 levels are elevated because of increased exocytosis, demonstrating an essential role of IRAP in GLUT4 retention. Current evidence supports the model that AS160 RabGAP, which is required for basal GLUT4 retention, is recruited to GLUT4 compartments via an interaction with IRAP. However, here we show that AS160 recruitment to GLUT4 compartments and AS160 regulation of GLUT4 trafficking were unaffected by IRAP knockdown. These results demonstrate that AS160 is recruited to membranes by an IRAP-independent mechanism. Consistent with a role independent of AS160, we showed that IRAP functions in GLUT4 sorting from endosomes to GLUT4-specialized compartments. This is revealed by the relocalization of GLUT4 to endosomes in IRAP knockdown cells. Although IRAP knockdown has profound effects on GLUT4 traffic, GLUT4 knockdown does not affect IRAP trafficking, demonstrating that IRAP traffics independent of GLUT4. In sum, we show that IRAP is both cargo and a key regulator of the insulin-regulated pathway.

INTRODUCTION

Insulin stimulates glucose uptake into adipose and muscle cells by inducing translocation of glucose transporter 4 (GLUT4) glucose transporters from intracellular compartments to the plasma membrane (PM; Huang and Czech, 2007; Antonescu *et al.*, 2009). Adipose and muscle cells have a specialized mechanism to retain GLUT4 intracellularly in the absence of insulin, thereby keeping the amount of GLUT4 in the PM and the flux of glucose into those cells low in the fasted state. In unstimulated cells, GLUT4 is actively sequestered away from the general recycling endosomes into GLUT4-specialized compartments (Karylowski *et al.*, 2004; Zeigerer *et al.*, 2004; Martin *et al.*, 2006). GLUT4 exocytosis to the PM from these specialized compartments is slow compared with general exocytosis from recycling endosomes (Yeh *et al.*, 1995; Zeigerer *et al.*, 2004). On insulin stimulation, GLUT4 exocytosis is accelerated (Jhun *et al.*, 1992; Satoh *et al.*, 1993; Li *et al.*, 2001; Zeigerer *et al.*, 2002; Karylowski *et al.*, 2004; Lizunov *et al.*, 2005; Martin *et al.*, 2006) and in adipocytes also GLUT4 internalization is reduced (Jhun *et al.*, 1992; Blot and McGraw, 2008). The changes in GLUT4 exocytosis and endocytosis both contribute to a net increase of GLUT4 in the PM of insulin-stimulated adipocytes and muscle cells.

GLUT4 retention is established by its targeting to two different specialized compartments that depend on the functions of two cytoplasmic motifs: an NH_2-terminal $FQQI^{5-8}$ motif and COOH-terminal $TELE^{487-490}$-based motif (Govers *et al.*, 2004; Blot and McGraw, 2008; Xiong *et al.*, 2010). The $FQQI^{5-8}$ motif controls the sorting of GLUT4 from general endosomes to a perinuclear GLUT4 storage compartment from which GLUT4 cannot reach the PM directly. The $TELE^{487-490}$-based motif controls the sorting of GLUT4 from general endosomes to GLUT4-specialized vesicles (GSV) that ferry GLUT4 to the PM (Blot and McGraw, 2008). Mutation of either of these motifs leads to reduced targeting to the specialized retention pathway, enhanced localization of GLUT4 to endosomes, and a concomitant increase of GLUT4 in the PM (Govers *et al.*, 2004; Blot and McGraw, 2008; Xiong *et al.*, 2010). On the basis of the behaviors of the GLUT4 mutants, we favor a model in which GSV are not directly formed from the storage compartment. However, we cannot rule out there is direct communication between the two retention compartments.

The above data on the intracellular distributions of the GLUT4 mutants underlines the importance of proper sorting of GLUT4 to the specialized compartments in the retention of GLUT4. Yet, the full complement of proteins involved in the specialized recognition and sorting of GLUT4 is un-

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† These authors contributed equally to this work.

Address correspondence to: Timothy E. McGraw (temcgraw@med.cornell.edu).

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known. One of the proteins required for basal GLUT4 retention is the Rab GTPase-activating protein AS160 (Sano *et al.*, 2003; Zeigerer *et al.*, 2004; Eguez *et al.*, 2005; Larance *et al.*, 2005). AS160 controls the amount of GLUT4 in the PM by controlling the docking of GSV with the PM (Zeigerer *et al.*, 2004; Jiang *et al.*, 2008). In unstimulated cells AS160 is active and keeps its target Rab proteins in their inactive conformation (Miinea *et al.*, 2005; Ishikura *et al.*, 2007). AS160 is phosphorylated by Akt2 upon insulin stimulation (Sano *et al.*, 2003), which leads to its inactivation and allows activation of its substrate Rab proteins. In adipocytes, Rab10 is a main target of AS160 and is required for insulin-stimulated translocation of GLUT4 to the PM (Sano *et al.*, 2007).

The insulin-regulated aminopeptidase, IRAP, is a type II membrane protein (Keller *et al.*, 1995). The luminal domain of IRAP contains an aminopeptidase activity that is involved in peptide hormone processing and antigenic peptide processing (Wallis *et al.*, 2007; Saveanu *et al.*, 2009). In insulin-sensitive tissues IRAP traffics together with GLUT4 to the specialized compartments and, like GLUT4, is retained intracellularly in the basal state and translocates to the plasma membrane in an insulin-regulated manner (Keller *et al.*, 1995; Ross *et al.*, 1996, 1997; Garza and Birnbaum, 2000).

Although IRAP is clearly a cargo of the insulin-regulated trafficking pathway, there are several observations that suggest IRAP might have a role in GLUT4 trafficking. For example, in vivo studies in mouse models suggest there might be a functional interaction between GLUT4 and IRAP, because knockout of either of the proteins affects the stability of the other (Jiang *et al.*, 2001; Keller *et al.*, 2002; Abel *et al.*, 2004; Carvalho *et al.*, 2004). Moreover, recent studies showed there is an interaction between the cytoplasmic domain of IRAP and AS160, leading to the hypothesis that IRAP has a role in targeting AS160 to the GSV (Larance *et al.*, 2005; Peck *et al.*, 2006). Because AS160 is an essential element of the GLUT4 retention machinery, in this model IRAP, by virtue of recruitment of AS160 to GSV, is more than cargo of the specialized GLUT4 pathway and is also a critical component of GLUT4 retention machinery.

Here we used short hairpin RNA (shRNA) protein depletion to further define the role of IRAP in GLUT4 trafficking. IRAP-knockdown (KD) resulted in a two- to threefold increase in basal PM GLUT4, due to accelerated exocytosis that correlated with a partial redistribution of GLUT4 to rapidly recycling endosomes. These findings document an essential role of IRAP in basal GLUT4 retention by regulating GLUT4 sorting from endosomes to GSV. Although loss of IRAP affected GLUT4 behavior, GLUT4-KD did not affect IRAP trafficking. In addition, we found that AS160 association with and AS160 regulation of GLUT4 compartments was unaffected in IRAP-KD adipocytes. This demonstrates IRAP is not essential for the targeting of AS160 to membranes and indicates IRAP and AS160 have independent roles in basal GLUT4 retention.

MATERIALS AND METHODS

Antibodies and Reagents

Anti-hemagglutinin (HA) epitope antibody (HA.11) was purified from ascites (Covance, Berkley, CA) on a protein G affinity column (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Mouse monoclonal anti-human transferrin receptor was a gift from Dr. Fred Maxfield (Weill Cornell Medical College) and rabbit anti-GLUT4 was a gift from Dr. Sam Cushman (National Institutes of Health). Rabbit anti-IRAP was described earlier (Keller *et al.*, 1995). Rabbit anti-AS160 was purchased from Upstate Biotechnology (Lake Placid, NY), rabbit anti-FLAG from Sigma-Aldrich (St. Louis, MO), rabbit anti-actin from Cytoskeleton (Denver, CO), rabbit anti-PPARγ from Santa Cruz Biotechnology (Santa Cruz, CA). All fluorescence secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and the horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from Pierce Chemical (Rockford, IL; Thermo Fisher Scientific). Alexa^{546} was purchased from Invitrogen (Carlsbad, CA) and was conjugated to human transferrin (Sigma-Aldrich) according to the manufacturer's instructions.

Cell Culture, Electroporation, and Plasmids

3T3-L1 fibroblast were differentiated and electroporated as described previously (Zeigerer *et al.*, 2002). Studies were performed 1 d after electroporation, with the exception of the transient knockdowns. Wild-type (WT) HA-GLUT4-GFP and the FA and EEAA mutants have been described previously (Lampson *et al.*, 2001; Blot and McGraw, 2006, 2008). The IRAP rescue construct was made by cloning IRAP from the pCR Script (SK+; Keller *et al.*, 1995) into pCDNA3.1 (+) (Invitrogen) using BamHI and HindIII sites. The wobble mutations were created in the target sides of the shRNA using QuickChange mutagenesis XL (Stratagene, La Jolla, CA) following manufacturer's instructions. pCMV FLAG-AS160 WT and 4P were previously described (Zeigerer *et al.*, 2004; Eguez *et al.*, 2005). pCMV transferrin (Tf) receptor (TR) and pCMV IRAP-TR (a fusion of the first 109 amino acids of IRAP with the transmembrane and luminal domains of TR) have been described previously (Johnson *et al.*, 1998).

Transient and Stable Knockdown

For transient knockdown experiments 2 nmol of stealth small interfering RNA (siRNA; Invitrogen) against mouse AS160 was added to each electroporation as described earlier (Eguez *et al.*, 2005). Experiments were performed 2 d after electroporation. For stable knockdown of IRAP and GLUT4, shRNAs were designed against the luminal domain of mouse IRAP (GGCTGGTTGTTCCTCTTT; base pairs 2617–2634) and GLUT4 (GGTGATTGAACAGAGCTAC; base pairs 149–167) using the pSIREN RetroQ system (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Phoenix packaging cells were transfected with the retroviral cDNA, and culture medium from the packaging cells, harvested between 24 and 48 h after transfection, was used to infect 3T3-L1 cells (Wertheim *et al.*, 2004). The infected cells were selected for growth in puromycin, the surviving cells were differentiated, and the behavior of GLUT4 or IRAP-TR was characterized. The control retrovirus expressing a shRNA not targeting any mouse gene has been described previously (Eguez *et al.*, 2005).

Translocation Assay

All live cell incubations were performed in serum-free DMEM with 20 mM sodium bicarbonate, 20 mM HEPES (pH 7.2; SF-DMEM) at 37°C in 5% CO_2. In all experiments, adipocytes were preincubated in SF-DMEM medium for at least 120 min at 37°C in 5% CO_2/air (basal conditions). For insulin stimulation, cells were incubated in SF-medium containing 1 nM insulin (Sigma-Aldrich) for 30 min at 37°C in 5% CO_2. Cells were washed twice in ice-cold PBS containing Ca^{2+}/Mg^{2+} and fixed in 3.7% formaldehyde (Sigma). To measure GLUT4 translocation, cells expressing HA-GLUT4-GFP were stained for surface HA.11 in nonpermeabilized cells using indirect immunofluorescence with Cy3-labeled goat anti-mouse IgG, whereas green fluorescent protein (GFP) fluorescence is a measure of total HA-GLUT4-GFP per cell. To detect expression of IRAP or FLAG-AS160 constructs, cells were permeabilized with 0.025% saponin and subjected to indirect immunofluorescence using either rabbit anti-IRAP or rabbit anti-FLAG.

To measure the translocation of TR and IRAP-TR, cells electroporated with cDNA encoding human TR or IRAP-TR were treated overnight with an antibody against mouse TR to block endogenous TR expression. The next day cells were incubated in serum-free DMEM containing Tf-Alexa^{546} for at least 3 h. Then the total amount of TR expressed per cell is determined by the uptake of Tf-Alexa^{546}, whereas the amount of TR at the surface was determined using an antibody against the extracellular domain of human TR in nonpermeabilized cells.

Kinetic Assays

The exocytosis rate constants of HA-GLUT4-GFP in basal adipocytes were determined as described previously (Karylowski *et al.*, 2004; Blot and McGraw, 2008). We incubated HA-GLUT4-GFP–expressing adipocytes with saturating amount of anti-HA.11 for 5, 30, 60, 120, 200, 240, or 420 min, respectively. Cells were fixed and internalized anti-HA.11 was revealed by indirect immunofluorescence using Cy3-labeled goat anti-mouse IgG in saponin-permeabilized cells The ratio of cell-associated Cy3 to total HA-GLUT4-GFP versus time ± SEM was plotted, and each data point from the individual experiments was normalized to the control plateau.

The internalization rate of HA-GLUT4-GFP in basal adipocytes was measured by incubating HA-GLUT4-GFP–expressing cells with a saturating amount of anti-HA.11 in the serum-free DMEM for 5, 10, 20, or 30 min at 37°C. Cells were fixed, and HA.11 on the surface was blocked using saturating concentrations of Cy5-conjugated secondary antibodies without permeabilization. To reveal internalized HA.11, cells were saponin-permeabilized and

stained with Cy3-labeled goat anti-mouse IgG. The ratio of internalized anti-HA to average surface HA-GLUT4-GFP for each time point was determined and fit to linear curves, the slopes of which are a measure of the relative internalization rate of basal HA-GLUT4-GFP.

Determining the Membrane-bound Fraction of AS160

Adipocytes expressing both HA-GLUT4-GFP and FLAG-AS160 were starved for 2 h in serum-free DMEM. Cells were washed with cold PBS supplemented with Ca^{2+}/Mg^{2+} and subsequently permeabilized for 1 min in 80 mM Pipes containing 10 mM EGTA, 1 mM $MgCl_2$, and 0.05% saponin to leak out cytosolic contents (Gonzalez and McGraw, 2009). AS160 was revealed by indirect immunofluorescence using rabbit anti-FLAG in saponin-permeabilized cells. HA-GLUT4-GFP was used as an expression marker to identify electroporated cells. For each sample, the average AS160 fluorescence intensity was determined, and the membrane-bound fraction of AS160 was determined by the ratio of AS160 fluorescence intensity in permeabilized versus nonpermeabilized cells.

Ablation Assay

The intracellular distribution of HA-GLUT4-GFP between endosomes and GSV was measured using the epitope ablation assay as described previously (Johnson *et al.*, 2001; Zeigerer *et al.*, 2002; Karylowski *et al.*, 2004). In short, cells expressing both HA-GLUT4-GFP and human TR were incubated in media containing saturating levels of anti-HA.11 and with or without Tf-HRP for 3–4 h. Subsequently, cells were placed on ice and incubated for 30 min with 250 μg/ml diaminobenzidine (DAB) and 0.0025% H_2O_2, which ablates all HA.11 epitopes present in the same compartment as Tf-HRP. Cells were fixed with 3.7% formaldehyde and the HA.11 epitopes that were not ablated were revealed by indirect immunofluorescence in saponin-permeabilized cells using Cy3-labeled goat anti-mouse IgG. The difference in the Cy3 fluorescence between cells with versus without Tf-HRP represents the fraction of total HA-GLUT4-GFP that was present in Tf-positive endosomes.

Immunoabsorption and Immunoblotting

For immunoabsorption, electroporated HA-GLUT4-GFP control or IRAP-KD adipocytes were washed once in PBS, scraped into 1 ml of HES buffer (20 mM HEPES, 1 mM EDTA, 250 mM sucrose, and protease inhibitors) and homogenized by subsequent passaging through Q18G1/4 and Q26G5/8 syringes on ice. Total cell homogenates were cleared by centrifugation at 1300 $\times$ *g* for 7 min. GLUT4-containing compartments were isolated by incubation with GFP beads, according to manufacturer's instructions (Miltenyi Biotech, Bergish Gladbach, Germany). For total cell lysates, cells were washed in PBS and lysed in Laemmli buffer. Lysates were sheared through a Q26G5/8 syringe, and proteins were resolved in SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against AS160 and actin according to the supplier's protocols. Protein contents were quantified by Odyssey Li-Cor software (Lincoln, NE) or ImageJ software (http://rsb.info.nih.gov/ij/).

Data Acquisition and Processing

Fluorescent images were collected on a DMIRB inverted microscope (Leica Microsystems, Deerfield, IL) coupled to a charge-coupled device 12-bit camera (Princeton Instruments, West Chester, PA) using a 40$\times$ 1.25 NA oil-immersion objective. Fluorescence quantifications were done using MetaMorph image processing software (Molecular Devices, Sunnyvale, CA) as described previously (Lampson *et al.*, 2000, 2001; Zeigerer *et al.*, 2002).

RESULTS

IRAP Is Required for Basal GLUT4 Retention

To investigate the role of IRAP in GLUT4 trafficking, we stably knocked down IRAP in 3T3-L1 adipocytes. This knockdown resulted in an approximate 90% reduction in IRAP expression (Figure 1A). IRAP-KD did not affect proteins whose expression is increased with differentiation of adipocytes, including the transcription factor PPARγ and Rab GTPase-activating protein AS160 (Figure 1, A and B). The expression of GLUT4, which is also up-regulated during differentiation, was not markedly affected by IRAP-KD (Figure 1, A and B). These data suggest that IRAP expression is not essential for differentiation of preadipocytes into adipocytes.

Intracellular GLUT4 is distributed between a perinuclear compartment and punctate structures distributed throughout the cytoplasm, a distribution that was not grossly altered by IRAP-KD (Figure 1C). We used a GLUT4 reporter with an exofacial HA epitope and GFP fused to its carboxyl-terminus to quantify the effect of IRAP knockdown on GLUT4 distribution between intracellular compartments and the PM. Total HA-GLUT4-GFP is based on GFP fluorescence, whereas HA-GLUT4-GFP inserted into the PM is revealed by anti-HA immunofluorescence in nonpermeabilized cells (Lampson *et al.*, 2000). In the IRAP-KD adipocytes there was an increase in anti-HA staining, demonstrating that IRAP-KD resulted in an increase of HA-GLUT4-GFP in the PM of basal adipocytes (Figure 1D). Quantification of the distribution of HA-GLUT4-GFP between the PM and intracellular compartments revealed a two- to threefold increase of PM GLUT4 in basal IRAP-KD adipocytes (Figure 1E). Transient reexpression of IRAP in IRAP-KD adipocytes, which resulted in an expression level of about three times that of endogenous IRAP, completely restored basal GLUT4 retention (Figure 1E). This confirms that the increased PM GLUT4 in basal IRAP-KD adipocytes was due to a loss of IRAP.

Insulin stimulated a large translocation of HA-GLUT4-GFP to the PM of IRAP-KD adipocytes, demonstrating that GLUT4 distribution was still under the control of insulin in these cells (Figure 1E). Translocation in IRAP-KD adipocytes tended to be smaller than in control adipocytes, although this difference did not achieve statistical significance ($p = 0.45$). Transient reexpression of IRAP in the IRAP-KD adipocytes increased the translocation of GLUT4 to the PM of IRAP-KD adipocytes, an effect that also did not reach statistical significance ($p = 0.08$). Although these data may suggest a possible role for IRAP in controlling the behavior of GLUT4 in the insulin-stimulated cells, the predominant effect of IRAP-KD was the reduced basal GLUT4 intracellular retention.

To determine whether IRAP-KD affected general membrane trafficking, we examined the TR distribution between the PM and intracellular compartments. IRAP-KD did not have an effect on either the basal or the insulin-stimulated distribution of the TR, demonstrating that IRAP's role is specific to GLUT4 trafficking (Figure 1F).

The Cytoplasmic Domain of IRAP Is Sufficient for Its Role in Basal GLUT4 Retention

Previous studies have documented that the IRAP cytoplasmic domain is sufficient for targeting IRAP to the specialized GLUT4-trafficking pathway (Lampson *et al.*, 2000; Subtil *et al.*, 2000; Johnson *et al.*, 2001; Hou *et al.*, 2006). To determine which domain of IRAP is involved in GLUT4 basal retention, we made use of IRAP-TR, a construct that contains the cytoplasmic domain of IRAP fused to the transmembrane and extracellular domains of the TR (Johnson *et al.*, 1998). Transient expression of IRAP-TR, which reached expression levels of three times that of endogenous IRAP, rescued the increase in basal PM GLUT4 to the same degree as reexpression of full-length IRAP (Figure 1E). Expression of IRAP-TR in IRAP-KD adipocytes had no significant effect on the insulin-stimulated GLUT4 translocation (Figure 1E). These results demonstrate that the IRAP cytoplasmic domain is sufficient for IRAP function in basal GLUT4 retention, thereby eliminating a role for the IRAP transmembrane and luminal/extracellular domains in this process.

IRAP Traffics Independent of GLUT4

Mice knockout models indicated that the absence of either GLUT4 or IRAP affects the stability and/or trafficking of the other protein, supporting the hypothesis that GLUT4 and IRAP are functionally linked (Jiang *et al.*, 2001; Keller *et al.*, 2002; Abel *et al.*, 2004; Carvalho *et al.*, 2004). Our data that GLUT4 distribution is dependent on the presence of IRAP is

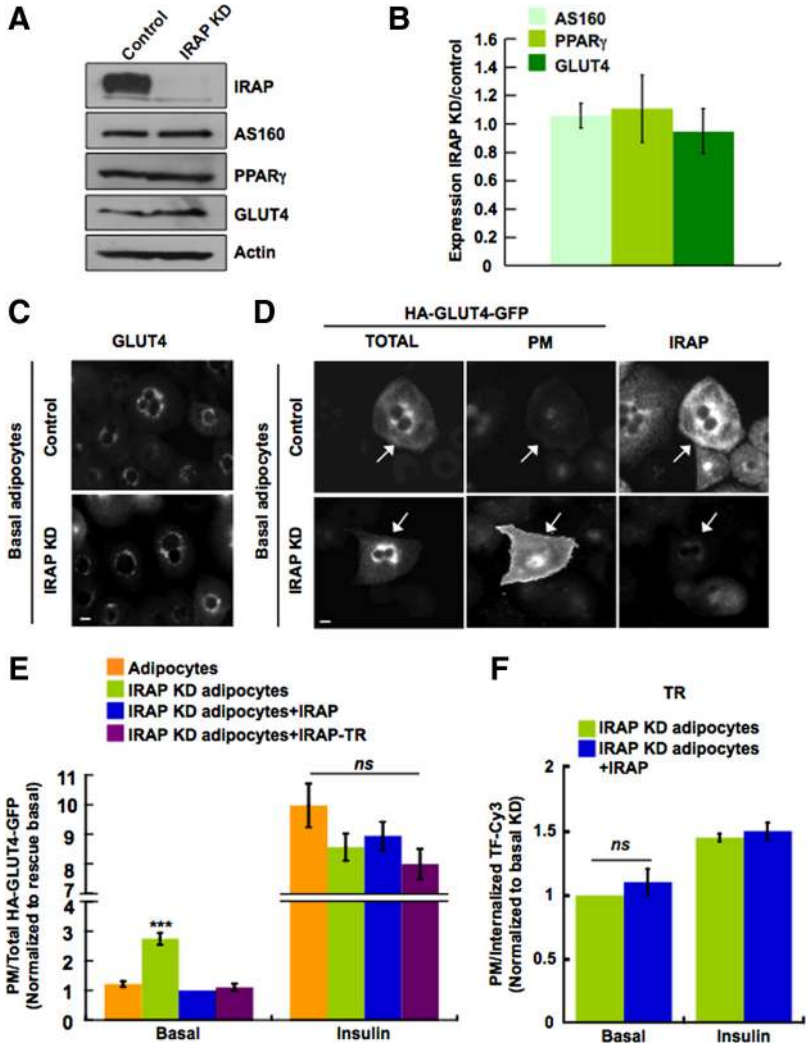

Figure 1. IRAP is essential for basal GLUT4 intracellular retention. (A) Representative immunoblot analysis of the indicated differentiation markers in cell extracts of control 3T3-L1 adipocytes and IRAP-KD adipocytes at day 5 after differentiation. (B) Quantification of the expression of the indicated differentiation markers normalized to actin as the ratio IRAP-KD adipocytes-to-control adipocytes. Each data point represents the average ± SEM of 5–7 independent experiments. (C) Epifluorescence images detecting endogenous GLUT4 in basal control and IRAP-KD adipocytes. Bar, 10 μm. (D) Epifluorescence images of basal control and IRAP-KD adipocytes expressing HA-GLUT4-GFP. Surface GLUT4 (PM) and endogenous IRAP were detected by indirect immunofluorescence of anti-HA and anti-IRAP, respectively. Arrows indicate HA-GLUT4-GFP–expressing cells. Bar, 10 μm. (E) PM-to-total HA-GLUT4-GFP ratios of control, IRAP-KD, and IRAP-KD adipocytes reexpressing IRAP, or IRAP-TR in basal and insulin-stimulated state. Each data point represents the average ± SEM of 8–20 experiments. The data from the individual experiments were normalized to basal IRAP-KD adipocytes reexpressing IRAP (rescue basal). Insulin-stimulated cells were incubated with 1 nM insulin for 30 min at 37°C. IRAP or IRAP-TR expression was verified by indirect immunofluorescence in saponin-permeabilized cells (***$p < 0.0001$ compared with control; ns, nonsignificant, paired Student's t test). (F) PM-to-total transferrin receptor (TR) ratios of IRAP-KD adipocytes or IRAP-KD adipocytes reexpressing IRAP in basal and insulin-stimulated state. Each data point represents the average ± SEM of 2–3 experiments (ns, nonsignificant; paired Student's t test). The data from the individual experiments are normalized to basal IRAP-KD adipocytes (Basal KD). IRAP expression was verified by indirect immunofluorescence.

consistent with this hypothesis. We next tested whether the reverse was true by studying the behavior of IRAP-TR in GLUT4-KD adipocytes. The level of GLUT4 was reduced by ~80% in GLUT4-KD adipocytes (Figure 2A). GLUT4-KD adipocytes differentiated normally based on lipid droplet formation, and the reduction in GLUT4 did not affect the intracellular localization of endogenous IRAP (Figure 2, B and C). The distribution of IRAP-TR between the PM and intracellular compartments was not changed by GLUT4-KD (Figure 2D), indicating IRAP traffics independent of GLUT4.

Transient knockdown of AS160, a known regulator of the GLUT4/IRAP pathway (Sano *et al.*, 2003; Zeigerer *et al.*, 2004; Eguez *et al.*, 2005; Larance *et al.*, 2005), resulted in a twofold increase in basal PM IRAP-TR and a blunting in insulin-stimulated translocation in both control and GLUT4-KD adipocytes (Figure 2E). These changes are in the same range as we observed for the effects on GLUT4, indicating that effects on trafficking of IRAP-TR will be revealed using this assay. These above results also demonstrate that the AS160-dependent, insulin-regulated pathway is functional in the GLUT4-KD adipocytes. Therefore, GLUT4, in contrast to IRAP, does not have a role in the function of the insulin-regulated pathway and therefore is only cargo of the insulin-regulated pathway.

IRAP-KD Increased Basal GLUT4 Exocytosis without Affecting Internalization

We next investigated the underlying changes in GLUT4 behavior that cause the increased PM GLUT4 in IRAP-KD adipocytes. The amount of GLUT4 in the PM is determined by its rates of exocytosis and endocytosis (Karylowski *et al.*, 2004). In IRAP-KD adipocytes HA-GLUT4-GFP exocytosis was about twofold faster than in control adipocytes, a change that accounts for the increased accumulation of GLUT4 in the PM (Figure 3A). This increased exocytosis was rescued by reexpression of either full-length IRAP or IRAP-TR (Figure 3A). In fact, GLUT4 exocytosis in IRAP-KD adipocytes reexpressing IRAP or expressing IRAP-TR was slower than in control adipocytes, suggesting IRAP levels are limiting in unstimulated adipocytes (Figure 3A). To test this further, we overexpressed either IRAP or IRAP-TR in control adipocytes to about three times that of the endogenous IRAP. This resulted in a reduction in the exocytosis of GLUT4 (Figure 3B) and reduced basal steady state PM GLUT4 in control adipocytes by ~30%, confirming the important role of exocytosis in establishing the amount of PM GLUT4 in basal adipocytes (Figure 3C). The effects of overexpression of IRAP on GLUT4 were limited to basal conditions since insulin-stimulated redistribution of GLUT4 to the

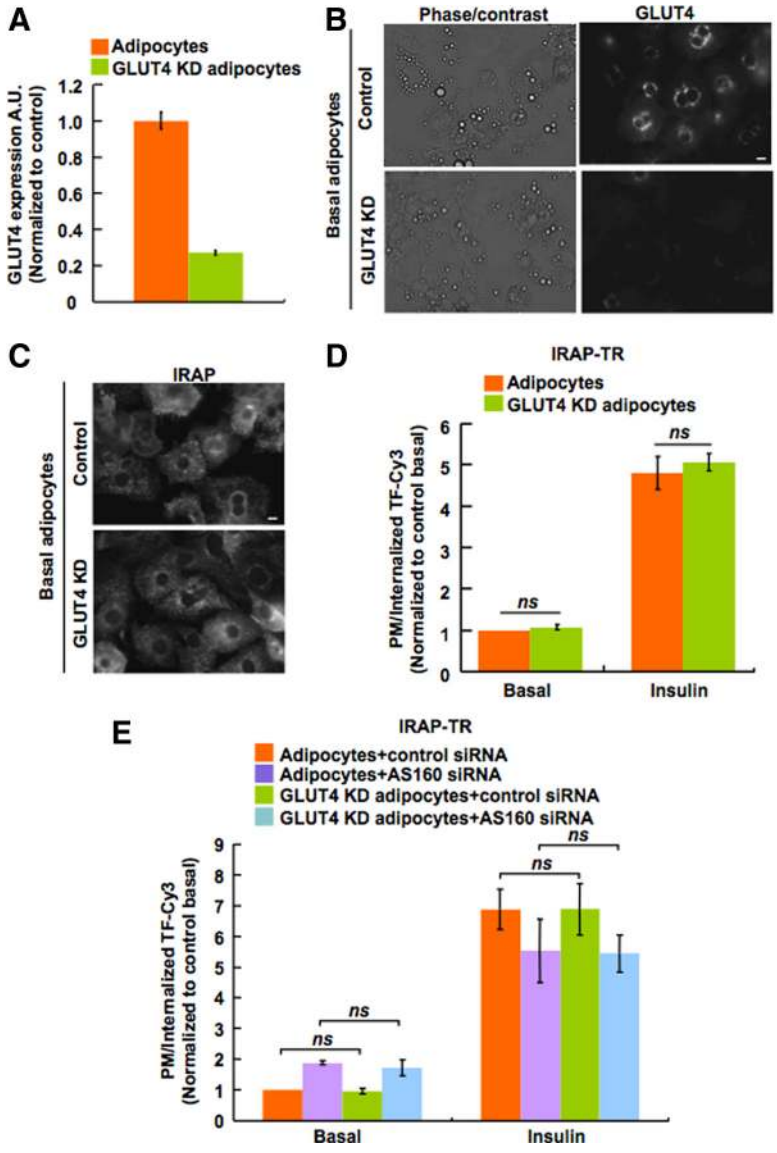

Figure 2. IRAP traffics independent of GLUT4. (A) Quantification of the expression of endogenous GLUT4 detected by indirect immunofluorescence in control and GLUT4-KD adipocytes in arbitrary units (A.U.). Each data point represents the average ± SEM; n = > 50 cells. (B) Phase-contrast and epifluorescence images of basal control and GLUT4-KD adipocytes. Endogenous GLUT4 was detected by indirect immunofluorescence with GLUT4 antibodies. Bar, 10 μm. (C) Epifluorescence images of basal control and GLUT4-KD adipocytes. Endogenous IRAP was detected by indirect immunofluorescence with IRAP antibodies. Bar, 10 μm. (D) PM-to-total IRAP-TR ratios of control and GLUT4-KD adipocytes in basal and insulin-stimulated states. Each data point represents the average ± SEM of three experiments. The data from the individual experiments were normalized to basal control adipocytes. Insulin-stimulated cells were incubated with 1 nM insulin for 30 min at 37°C. ns, nonsignificant; paired Student's *t* test. (E) Control and GLUT4-KD adipocytes were transfected with either control siRNA or AS160 siRNA. Data points represent PM-to-total IRAP-TR distributions in basal and insulin-stimulated state of three independent experiments ± SEM. Insulin-stimulated cells were incubated with 1 nM insulin for 30 min at 37°C. ns, nonsignificant; paired Student's *t* test.

PM was unaltered by overexpression of IRAP (Figure 3C). These data suggest IRAP levels are limiting in basal adipocytes.

Neither IRAP-KD nor its overexpression affected GLUT4 internalization (Figure 3D). This indicates that the two- to threefold increase in basal surface GLUT4 we observed in IRAP-KD adipocytes is due increased GLUT4 exocytosis, documenting that IRAP has a specific and essential role in GLUT4 exocytosis in basal adipocytes.

IRAP and AS160 Function at Different Steps of the Retention Mechanism

AS160 RabGAP is a well-studied regulator of the specialized GLUT4 trafficking pathway. In basal adipocytes, AS160 inhibits GLUT4 insertion into the PM by specifically inhibiting a prefusion step of GSV with the PM (Zeigerer *et al.*, 2004; Jiang *et al.*, 2008). Knockdown of AS160 partially releases GLUT4 basal retention without affecting the distribution of TR (Eguez *et al.*, 2005; Larance *et al.*, 2005). Thus, the effect of IRAP-KD on the basal GLUT4 distribution is similar to AS160 knockdown. It has recently been shown that the cytoplasmic domain of IRAP interacts with AS160, suggesting that IRAP recruits AS160 to GLUT4-containing vesicles (Larance *et al.*, 2005; Peck *et al.*, 2006). Therefore, one possibility for the similarities in the phenotypes of AS160 and IRAP-KDs, is that IRAP-KD impairs AS160 recruitment to GLUT4 vesicles, leading to increased basal exocytosis and a concomitant increase in PM GLUT4. If this is the case, simultaneous knockdown of AS160 and IRAP would not have additive effects on GLUT4.

We performed a transient knockdown of AS160 in IRAP-KD adipocytes (Figure 4A). In control adipocytes, transient AS160 knockdown resulted in a twofold increase in basal PM GLUT4, consistent with previous data (Figure 2E; [Eguez *et al.*, 2005]). In IRAP-KD adipocytes, AS160 knockdown resulted in an additive twofold increase in basal PM GLUT4, with about a net fivefold increase of GLUT4 in the PM of AS160/IRAP double knockdown basal adipocytes. Reexpression of IRAP in AS160/IRAP double knockdown adipocytes restored PM GLUT4 to the level of AS160 knockdown alone (Figure 4A). These results show there was additivity of effects when AS160 and IRAP were simulta-

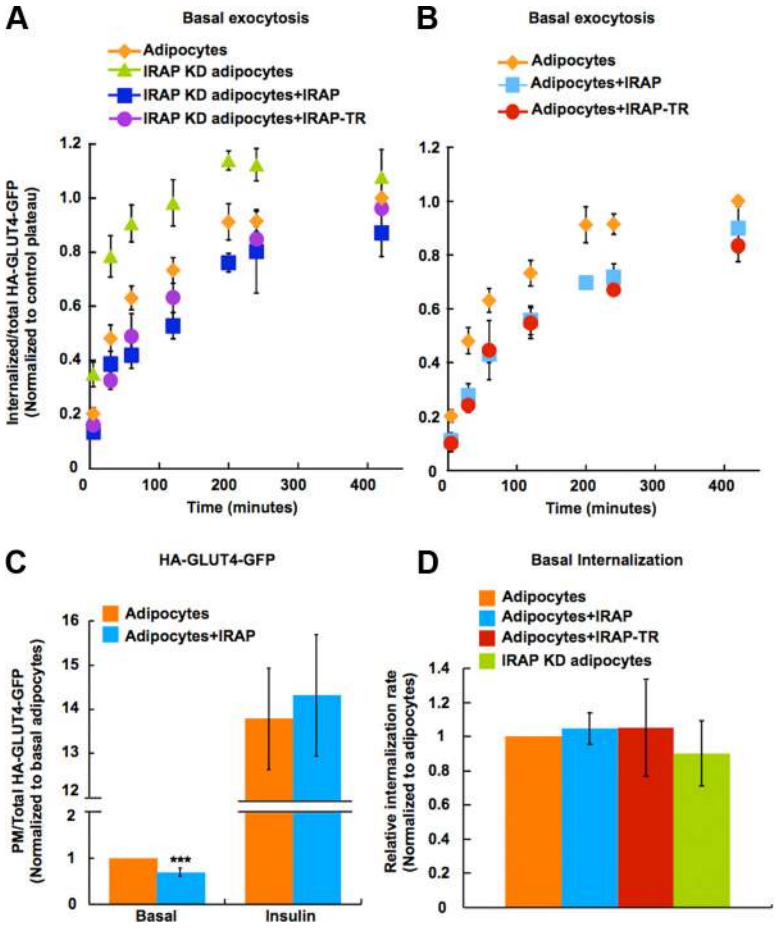

Figure 3. Effects of IRAP-KD and IRAP overexpression on HA-GLUT4-GFP exocytosis and internalization. HA-GLUT4-GFP exocytosis in (A) basal control adipocytes, IRAP-KD adipocytes, IRAP-KD adipocytes reexpressing IRAP or IRAP-TR, and (B) control adipocytes and control adipocytes expressing IRAP or IRAP-TR. Data from at least five different experiments was averaged and plotted as the ratio internalized-to-total HA-GLUT4-GFP versus time ± SEM. Each data point from the individual experiments was normalized to the control plateau. In this assay the time to achieve the plateau reflects the exocytosis rate constant. (C) PM-to-total HA-GLUT4-GFP ratios of control adipocytes and adipocytes overexpressing IRAP in basal and insulin-stimulated state. Each data point represents the average ± SEM of 4–10 experiments (***$p < 0.001$ compared with basal control; paired Student's *t* test). The data from the individual experiments are normalized to basal control. IRAP expression was verified by indirect immunofluorescence. (D) Internalization of HA-GLUT4-GFP in basal control adipocytes, IRAP-KD adipocytes and control adipocytes expressing IRAP or IRAP-TR. IRAP coexpression with HA-GLUT4-GFP was verified by indirect immunofluorescence.

neously knocked down and thus indicate that IRAP and AS160 function at different steps of the GLUT4 retention pathway.

These findings provoked us to reconsider the hypothesis that IRAP targets AS160 to GLUT4-containing vesicles. The IRAP-KD adipocytes provide a useful tool to study the role of IRAP in membrane recruitment of AS160 and therefore we analyzed the localization of AS160 in IRAP-KD adipocytes. First, we measured membrane-associated AS160 in cells using a quantitative microscopy assay in which cells are saponin-permeabilized before fixation to allow soluble cytosolic contents to diffuse from cells (Schenck *et al.*, 2008; Gonzalez and McGraw, 2009). We expressed HA-GLUT4-GFP together with FLAG-AS160 and quantified the amounts of FLAG-AS160 with and without permeabilization in basal control and IRAP-KD adipocytes. The amount of FLAG-AS160 in each condition was determined, and the membrane-bound fraction was measured as the ratio of AS160 fluorescence in permeabilized cells to nonpermeabilized cells (Figure 4B). We did not observe differences in FLAG-AS160 membrane association between basal control and IRAP-KD adipocytes. We did see a release of FLAG-AS160 from membranes in control adipocytes stimulated with insulin, which did not change upon IRAP-KD (data not shown). These findings are consistent with previous reports that insulin promotes the release of AS160 from membranes (Larance *et al.*, 2005; Gonzalez and McGraw, 2009). This assay, however, measures AS160 on all membranes and is insensitive to shifts in the distribution of AS160 among different membranes. Therefore, we performed an immunoabsorption assay to isolate the GLUT4 compartments. Basal control and IRAP-KD adipocytes expressing HA-GLUT4-GFP were homogenized and the GLUT4-containing compartments were absorbed using beads coupled to anti-GFP antibody. No differences were observed between the amount of endogenous AS160 immunoabsorbed with GLUT4-containing compartments from control and IRAP-KD adipocytes (Figure 4C). Together these results demonstrate that although IRAP and AS160 might interact, this interaction is not required for the membrane localization of AS160.

IRAP Is Required for GLUT4 Sorting to GSV

The above data show that IRAP-KD does not affect AS160 recruitment to the membrane and therefore suggest that AS160 and IRAP control different steps in the GLUT4 retention pathway. We next tested whether IRAP could play a role in the sorting of GLUT4 to the storage compartment and/or GSV (Figure 5A, steps 1 and 2). The $FQQI^{5-8}$ is required for GLUT4 targeting to the GLUT4 storage compartments, from which GLUT4 is unable to directly traffic to the plasma membrane (Figure 5A, step 2; Blot and McGraw, 2008). The $TELE^{487-490}$-based motif functions in the dynamic retention cycle between the endosomes and the GSV that ultimately dock and fuse with the PM in the presence of insulin (Figure 5A, step 1; Govers *et al.*, 2004; Blot and McGraw, 2008). In previous studies using mutants of these trafficking motifs we have shown that reduced sorting of GLUT4 from endosomes to the GLUT4-storage compartments and the GSV (Figure 5A, steps 1 and 2) results in an increase of exocytosis and concomitant increase in basal PM GLUT4 (Blot and McGraw, 2008; Xiong *et al.*, 2010), similar

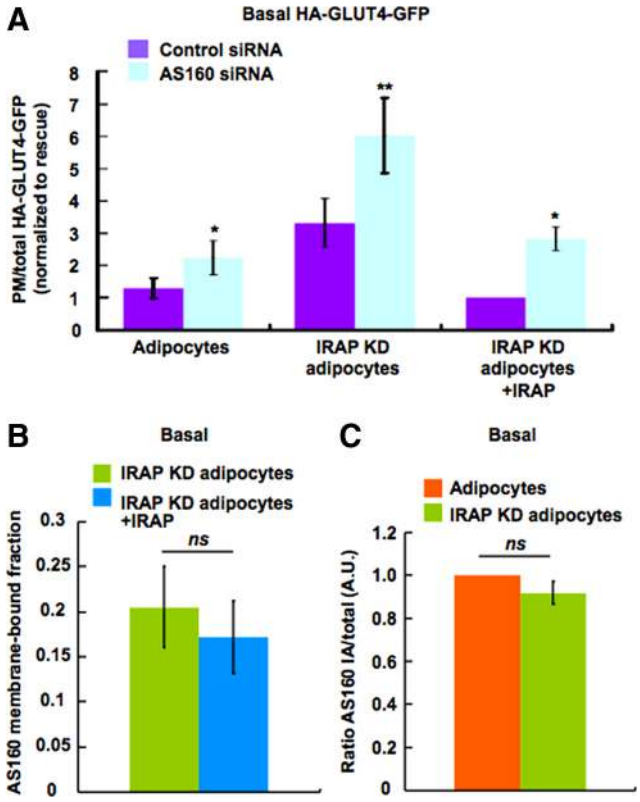

Figure 4. IRAP and AS160 function at different steps in GLUT4 intracellular retention. (A) Transient AS160 knockdown in basal IRAP-KD adipocytes has additive effects on PM GLUT4 levels. Control and IRAP-KD adipocytes were transfected with either control siRNA or AS160 siRNA. Data points represent the average basal PM-to-total HA-GLUT4-GFP distributions of four independent experiments ± SEM (*$p < 0.05$ compared with control siRNA, **$p < 0.001$ compared with control siRNA; paired Student's *t* test). The data from the individual experiments are normalized to IRAP-KD adipocytes reexpressing IRAP transfected with control siRNA (rescue). IRAP coexpression with HA-GLUT4-GFP was verified by indirect immunofluorescence. (B) IRAP-KD has no effect on the membrane localization of FLAG-AS160. Control and IRAP-KD adipocytes coexpressing HA-GLUT4-GFP and FLAG-AS160 were permeabilized, or not, before fixation. FLAG-AS160 was revealed by indirect immunofluorescence using rabbit anti-FLAG. Shown is membrane-bound fraction of FLAG-AS160 as the ratio of FLAG-AS160 fluorescence in permeabilized cells to nonpermeabilized cells. Each data point is the average of three independent experiments ± SEM (ns, nonsignificant; paired Student's *t* test). (C) IRAP is not essential for AS160 localization to GLUT4-positive compartments. GLUT4-containing compartments were isolated by immunoabsorption of HA-GLUT4-GFP using beads conjugated to anti-GFP and immunoblotted for endogenous AS160. Shown is the quantification of the fraction of total AS160 in the immunoabsorption (IA) of three independent experiments in control and IRAP KD adipocytes. Each data point of the individual experiments was normalized to the ratio in control adipocytes. (ns, nonsignificant; paired Student's *t* test).

to the change in behavior of GLUT4 in the IRAP-KD adipocytes.

To directly assess the effect of IRAP-KD on GLUT4 sorting, we tested the distribution of GLUT4 in TR-containing recycling endosomes versus the specialized compartments. We used a previously described endosome ablation assay to determine the relative amount of GLUT4 in TR-containing endosomes in basal control and IRAP-KD adipocytes (Martin *et al.*, 1996; Johnson *et al.*, 2001; Zeigerer *et al.*, 2002). We found a significant increase in the fraction of GLUT4 present in the endosomes in the IRAP-KD adipocytes, which could be reversed by IRAP reexpression (Figure 5B). These results provide direct support for the hypothesis that IRAP has a role in GLUT4 retention by regulating the sorting of GLUT4 from the endosomes to the GLUT4 compartments.

To determine at which step in the retention pathway IRAP has a role, we examined the behaviors of GLUT4 mutated in the FQQI$^{5-8}$ (F^5A) or the TELE$^{487-490}$ ($E^{488}A$,$E^{490}A$) motifs in IRAP-KD adipocytes. As previously shown, in control adipocytes the F^5A mutation induced a fourfold increase in PM GLUT4, whereas the increase induced by the $E^{488}A$,$E^{490}A$ was about twofold (Figure 5C; Blot and McGraw, 2008). Interestingly, IRAP-KD was additive to the F^5A mutation but not to the $E^{488}A$,$E^{490}A$ mutation (Figure 5C). Transient reexpression of IRAP in the IRAP-KD adipocytes largely restored the amount F^5A-GLUT4 in the PM to the level observed when the F^5A-GLUT4 mutant is expressed in control adipocytes and restored PM levels of the $E^{488}A$,$E^{490}A$-GLUT4 to PM level of wild-type GLUT4 levels (Figure 5C). These results provide strong support for the hypothesis that IRAP has a role in the sorting of GLUT4 from endosomes to the GSV (Figure 5A, step 1).

The above data suggest that in the IRAP-KD adipocytes some GLUT4 returns to the PM in TR-containing vesicles formed from endosomes. A dominant-interfering mutant of AS160 (AS160-4P), which is not inactivated after insulin stimulation, inhibits insulin-stimulated GLUT4 translocation (Figure 5A, step 3; Sano *et al.*, 2003; Zeigerer *et al.*, 2004). The effect of AS160-4P is specific for GSV, and trafficking of the TR is unaffected by expression of AS160-4P (Sano *et al.*, 2003; Zeigerer *et al.*, 2004). If the increased PM GLUT4 in basal IRAP-KD adipocytes is due to a partial redistribution of GLUT4 to TR recycling vesicles, then expression of AS160-4P should not reduce basal PM GLUT4. When we expressed AS160-4P in IRAP-KD adipocytes, we did not observe a decrease in basal PM GLUT4 (Figure 5D). These data support the hypothesis that in IRAP-KD adipocytes the increased basal PM GLUT4 reflects exocytosis of GLUT4 in vesicles that are not under the control of AS160 (i.e., not inhibited by AS160-4P) and thereby support the hypothesis that IRAP has a role in the sorting of GLUT4 from endosomes to GSV that ferry GLUT4 to the PM in the presence of insulin. Expression of AS160-4P did inhibit insulin-stimulated redistribution of GLUT4 to the PM of IRAP-KD adipocytes, consistent with the hypothesis that loss of IRAP affects the efficiency, but does not completely block GLUT4 sorting to the GSV (Figure 5D).

DISCUSSION

Intracellular basal retention of GLUT4 requires sorting of GLUT4 away from the fast recycling endosomes to a specialized exocytic recycling pathway that slowly equilibrates with the PM in basal adipocytes (Yeh *et al.*, 1995; Karylowski *et al.*, 2004). Important details regarding the mechanism of the specialized sorting are unknown. Here, we show that IRAP is an essential element of the machinery that sorts GLUT4 from endosomes to GSV to control basal GLUT4 retention. A previous study using adipocytes transiently knocked down for IRAP showed an impairment of the insulin-mediated GLUT4 translocation (Yeh *et al.*, 2007). Although we see a trend toward a decrease in the insulin-mediated GLUT4 translocation in our stable IRAP-KD adipocytes, the primary effect is observed in the basal state (Figure 1E). In addition, we show IRAP is not required for

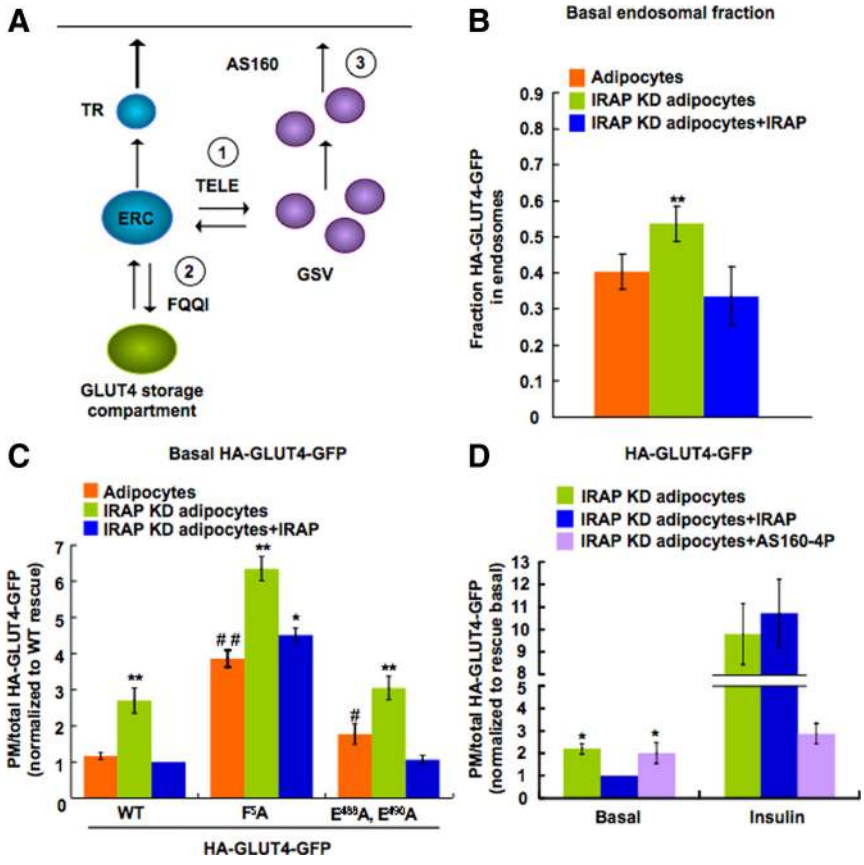

Figure 5. The intracellular distribution of GLUT4 in IRAP-KD adipocytes and the effect of IRAP-KD on the behavior of mutant GLUT4. (A) Schematic overview of the specialized GLUT4 pathway versus the general TR pathway and the steps at which IRAP could function. In basal cells GLUT4 is distributed to the specialized GLUT4 compartments and the general TR-positive endosomes. IRAP-KD can affect either the sorting of GLUT4 from the TR pathway to the GSV (step 1) regulated by the TELE motif, or the GLUT4 storage compartments (step 2) regulated by the FQQI motif, or affect the slow recycling specialized pathway (step 3), which is under control of AS160. (B) Distribution of HA-GLUT4-GFP between endosomes and GSV is affected by IRAP-KD. Each data point represents the average ± SEM of four independent experiments (**$p < 0.01$ compared with control; paired Student's *t* test). (C) The behaviors of the $FQQI^{5-8}$ and $TELE^{487-490}$ HA-GLUT4-GFP mutants in control and IRAP-KD adipocytes. PM-to-total HA-GLUT4-GFP WT, F^5A and $E^{488}A$,$E^{490}A$ distributions in basal control, IRAP-KD or IRAP-KD adipocytes reexpressing IRAP. Each data point represents the average ± SEM of 4–7 independent experiments (**$p < 0.01$ compared with own control adipocytes, *$p < 0.05$ compared with F^5A control adipocytes, $^{\#}p < 0.05$ compared with WT control, $^{\#\#}p < 0.01$ compared with WT control; paired Student's *t* test). The data from the individual experiments are normalized to WT HA-GLUT4-GFP in IRAP KD reexpressing IRAP adipocytes (WT rescue). IRAP expression was verified by indirect immunofluorescence. (D) The increase in basal PM GLUT4 in IRAP-KD adipocytes is insensitive to AS160-4P. PM-to-total distributions of HA-GLUT4-GFP in IRAP-KD adipocytes expressing IRAP or AS160-4P in basal and insulin-stimulated state. Shown is the average of four independent experiments ± SEM (*$p < 0.05$ compared with rescue basal; unpaired Student's *t* test). The data of the individual experiments is normalized to basal IRAP-KD reexpressing IRAP adipocytes (rescue basal). IRAP and AS160 expression were verified by indirect immunofluorescence.

the association of AS160 with GLUT4 compartments, indicating IRAP and AS160 have independent roles in the basal retention of GLUT4. Finally, we show that GLUT4, in contrast to IRAP, is just a cargo of the insulin-regulated trafficking pathway.

IRAP and AS160 Have Independent Roles in Basal GLUT4 Retention

In the basal state GSV fusion with the PM is inhibited by AS160 (Zeigerer *et al.*, 2004; Eguez *et al.*, 2005). AS160 functions by inactivating Rab10, which is required for the docking of GSV to the PM (Sano *et al.*, 2007). In the presence of insulin, Akt2 phosphorylates AS160 and thereby relieves this inhibition (Sano *et al.*, 2003), leading to GLUT4 translocation. Recent data showed that the membrane localization of AS160 to the GSV is essential for its function in basal GLUT4 retention (Stockli *et al.*, 2008). It is however largely unclear how AS160 targeting to the GSV is regulated. Data from two separate groups showed an interaction between AS160 and the cytoplasmic domain of IRAP (Larance *et al.*, 2005; Peck *et al.*, 2006), which led to the current hypothesis that IRAP is required for AS160 targeting to the GSV membrane. Our data do not support this hypothesis. First, we observed additivity in the increase in basal PM GLUT4 when AS160 and IRAP were simultaneously knocked down (Figure 4A). This would not be the case if IRAP-KD by itself would interfere with AS160 membrane targeting. Second, there was no effect on the amount of AS160 localization to GLUT4-positive compartments in IRAP-KD adipocytes (Figure 4C), which suggests IRAP is dispensable for membrane localization of AS160 and implicates other proteins in this process. A recently identified binding partner of AS160, the low-density lipoprotein receptor–related protein, might provide the binding site to recruit AS160 the GLUT4 vesicles (Jedrychowski *et al.*, 2009). A hypothesis that will need to be tested in future studies.

IRAP Is Required for Sorting GLUT4 to GSV That Have a Low Efflux Rate in Basal Adipocytes

Knockdown of IRAP resulted in an increased GLUT4 exocytosis to the PM. Our data suggest that the underlying cause of the increased exocytosis is the partial redistribution of GLUT4 from slowly recycling GSV to rapidly recycling general endosomes (Figure 5B). In this case, the GLUT4 exocytosis curves shown in Figure 3A and B consist of two components, a fast component that follows the efflux rate of TR and a slow component that follows the efflux rate of the GLUT4-specialized exocytosis vesicles. However, the data are equally well fit by a single or double exponential, which we interpret to indicate that the assay is not of sufficient sensitivity to detect the two components of exocytosis in the IRAP-KD adipocytes. Regardless, the increase in net GLUT4 exocytosis, the shift of GLUT4 to TR-positive endosomes, and the failure of dominant-interfering AS160-4P to reduce

basal PM GLUT4 in IRAP-KD adipocytes together provide compelling support for the hypothesis that the increased exocytosis is due to an increased fraction of GLUT4 returning to the PM via TR exocytic vesicles.

In addition to the finding that GLUT4 sorting to GSV in IRAP-KD adipocytes is less efficient, we also have data that suggest the opposite occurs upon overexpression of IRAP. In adipocytes overexpressing IRAP, we observed a slower exocytosis rate, which is reflected in a reduced basal PM GLUT4 (Figure 3, B and C). Therefore, overexpression of IRAP likely facilitates the sorting of GLUT4 away from the endosomes, thereby reducing the fast recycling fraction of GLUT4 in the endosomes. These results suggest that the amount of IRAP within an individual cell is limiting for sorting GLUT4 to the GSV.

The Role of the IRAP Cytoplasmic Domain in GLUT4 Sorting

Transient expression of the IRAP cytoplasmic domain, which contains all information necessary for the insulin-regulated trafficking of IRAP (Lampson *et al.*, 2000; Subtil *et al.*, 2000; Johnson *et al.*, 2001; Hou *et al.*, 2006), is sufficient to rescue the increased basal PM GLUT4 in IRAP-KD adipocytes (Figure 1E). Previous studies showed that microinjection of the IRAP cytoplasmic domain in adipocytes caused an insulin-independent translocation of GLUT4 to the PM (Waters *et al.*, 1997). The effect was dose-dependent, which suggests that the injected soluble cytoplasmic domain of IRAP interacts with proteins required for the specialized GLUT4-sorting machinery. Based on those findings, it was hypothesized that IRAP and GLUT4 might compete for the same sorting machinery. Our data demonstrate that IRAP and GLUT4 do not compete; on the contrary overexpression of IRAP further lowers the amount of GLUT4 at the surface in the basal state (Figure 3C). Therefore, it is more likely that the recruitment of the sorting machinery for GLUT4 is at least partially accomplished by IRAP. In this scenario, microinjected IRAP cytoplasmic domain, without a membrane-binding domain, competes for proteins normally recruited to membranes by endogenous IRAP, which would result in a phenotype resembling IRAP-KD. Several proteins have been identified to interact with the cytoplasmic domain of IRAP but only a couple have the properties expected of proteins involved in protein sorting and vesicle formation. These include p115, Tankyrase, and FHOS (formin homolog overexpressed in spleen; Chi and Lodish, 2000; Tojo *et al.*, 2003; Hosaka *et al.*, 2005). For future studies it will be of great interest to specify the different roles of these IRAP-interacting proteins in the function of IRAP in GLUT4 sorting.

IRAP and GLUT4 Are Not Essential for the Biogenesis of Insulin-regulated Compartments

GLUT4, IRAP, and Sortilin are part of the major cargo proteins of the GSV; however, how the GSV are originally formed and the order of events in this process is largely unclear. Previous studies suggested that Sortilin mediates the biogenesis of insulin-responsive compartments during differentiation, since the absence of Sortilin reduced the in vitro formation of these compartments, and adipocytes stably knocked down for Sortilin displayed impaired insulin-mediated GLUT4 translocation (Shi and Kandror, 2005, 2007). Our data indicate that IRAP is not essential for the formation of the insulin-responsive compartments. First, we are able to restore the basal GLUT4 retention by transient expression of either IRAP or its cytoplasmic domain in fully differentiated IRAP-KD adipocytes. Second, we show there is still formation of GSV in IRAP-KD adipocytes, given that GLUT4 in IRAP-KD adipocytes is still under the regulation of AS160, which is specific for the GSV (Zeigerer *et al.*, 2004; Eguez *et al.*, 2005). Therefore, we hypothesize that IRAP has a role after differentiation in the sorting of GLUT4 from the endosomal compartments to the specialized compartments, but is not essential for the original formation of the GSV.

In addition, we show that knockdown of GLUT4 has no effect on the insulin-responsiveness of IRAP-TR, indicating IRAP-TR still follows the insulin-regulated pathway in GLUT4-KD adipocytes. In the same cells IRAP-TR is still under the regulation of AS160, because transient knockdown of AS160 is able to reduce the basal retention (Figure 2E). These results suggest GLUT4 is cargo of the insulin-regulated pathway, but is not required for the formation of the insulin-regulated compartments and the sorting of IRAP-TR.

IRAP-KD Adipocytes versus the IRAP Knockout Mouse

The loss of IRAP on the behavior of GLUT4 has also been investigated using $IRAP^{-/-}$ mice. The most striking change in these mice was a dramatic reduction in GLUT4 protein levels in adipose cells, skeletal muscle, and heart (Keller *et al.*, 2002). This finding indicates that IRAP is required to maintain normal levels of GLUT4. We were, however, unable to recapitulate this decrease in endogenous GLUT4 expression in the stable IRAP-KD adipocytes (Figure 1B). Although many important discoveries regarding GLUT4 trafficking were revealed using adipocytes and muscle cell lines, these cultured cells may not fully represent the primary cells and tissues. One of the differences between primary and cultured adipocytes is the GLUT4 localization. In primary adipocytes most of the GLUT4 is localized to GLUT4-specialized compartments, whereas in cultured adipocytes only half of the GLUT4 is localized to the specialized compartments (Malide *et al.*, 1997). It has been shown that targeting of GLUT4 away from the endosomal system increases the half-life of GLUT4 (Shi and Kandror, 2005; Liu *et al.*, 2007), therefore redistribution of GLUT4 from the specialized compartments to general endosomes will have a larger effect on the GLUT4 stability in primary adipocytes compared with cultured adipocytes. Another possibility is that the change in GLUT4 levels in the $IRAP^{-/-}$ mouse do not reflect a direct effect of IRAP but rather a more complex compensatory change in response to increased basal GLUT4 trafficking to the cell surface and initially increased glucose uptake induced by IRAP knockout.

General Role for IRAP in Specialized Sorting

IRAP expression is not restricted to insulin-responsive tissues (Keller *et al.*, 1995; Rogi *et al.*, 1996). For example, in mast cells IRAP is found in a specialized compartment segregated from the general TR-positive recycling endosomes and upon stimulation with IgE, IRAP translocates to the PM in a manner independent of mast cell granula (Liao *et al.*, 2006).

The luminal aminopeptidase domain of IRAP, besides having a role in peptide hormone processing, has also been identified as the high-affinity binding site for Angiotensin IV and LVV-Hemorphin in the brain (Albiston *et al.*, 2001). Because agonists of IRAP are competitive inhibitors of its peptidase activity, it has been proposed that these compounds can improve memory by preventing processing of IRAP substrates that have a stimulatory effect on memory, including the recently identified substrate Vasopressin (reviewed in Albiston *et al.* (2003, 2007) and Wallis *et al.* (2007). Therefore it is tempting to speculate that neurons might have a sorting mechanism to keep IRAP intracellular to

prevent negative effects on memory under unstimulated conditions. In addition, in dendritic cells IRAP has been found in early phagosomes, where its aminopeptidase activity is involved in the processing of internalized antigens to facilitate MHC class I–mediated cross priming to CD8-positive T-cells (Saveanu *et al.*, 2009).

All the above cases concerned specialized secretory cells. Therefore, our study of IRAP's function in GLUT4 sorting in adipocytes could shed new light on the regulatory function of IRAP in cell types other than insulin-sensitive cells. IRAP could provide a general mechanism to prevent translocation of certain factors to the PM in the absence of stimulus by facilitating specialized sorting.

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