Insulin-stimulated exocytosis of GLUT4 is enhanced by IRAP and its partner tankyrase

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INTRODUCTION

Insulin modulates the exocytosis of diverse membrane proteins from the endosomal compartments to the PM (plasma membrane) in adipocytes. This effect is most prominent on the glucose transporter GLUT4 in adipocytes. This process is regulated by insulin-sensitive trafficking mechanisms, including its interaction with endogenous IRAP in GSVs. Recently, it was shown that knockdown of tankyrase, a Golgi-associated IRAP-binding protein that co-localizes with perinuclear GSVs, attenuated insulin-stimulated GSV translocation and glucose uptake without disrupting insulin-induced phosphorylation cascades. Moreover, iodoxanol density gradient analyses revealed that tankyrase knockdown altered the basal-state partitioning of GLUT4 and IRAP within endosomal compartments, apparently by shifting both proteins toward less buoyant compartments. Importantly, the aforementioned effects of tankyrase knockdown were reproduced by treating adipocytes with PJ34, a general PARP (poly-ADP-ribose polymerase) inhibitor that abrogated tankyrase-mediated protein modification known as poly-ADP-ribosylation. Collectively, these findings suggest that physiological GSV trafficking depends in part on the presence of IRAP in these vesicles, and that this process is regulated by tankyrase and probably its PARP activity.

Key words: adipocytes, GLUT4, GLUT1, insulin-responsive aminopeptidase (IRAP), iodoxanol gradients, tankyrase.

The glucose transporter GLUT4 and the aminopeptidase IRAP (insulin-responsive aminopeptidase) are the major cargo proteins of GSVs (GLUT4 storage vesicles) in adipocytes and myocytes. In the basal state, most GSVs are sequestered in perinuclear and other cytosolic compartments. Following insulin stimulation, GSVs undergo exocytic translocation to insert GLUT4 and IRAP into the plasma membrane. The mechanisms regulating GSV trafficking are not fully defined. In the present study, using 3T3-L1 adipocytes transfected with siRNAs (small interfering RNAs), we show that insulin-stimulated IRAP translocation remained intact despite substantial GLUT4 knockdown. By contrast, insulin-stimulated GLUT4 translocation was impaired upon IRAP knockdown, indicating that IRAP plays a role in GSV trafficking. We also show that knockdown of tankyrase, a Golgi-associated IRAP-binding protein that co-localizes with perinuclear GSVs, attenuated insulin-stimulated GSV translocation and glucose uptake without disrupting insulin-induced phosphorylation cascades. Moreover, iodoxanol density gradient analyses revealed that tankyrase knockdown altered the basal-state partitioning of GLUT4 and IRAP within endosomal compartments, apparently by shifting both proteins toward less buoyant compartments. Importantly, the aforementioned effects of tankyrase knockdown were reproduced by treating adipocytes with PJ34, a general PARP (poly-ADP-ribose polymerase) inhibitor that abrogated tankyrase-mediated protein modification known as poly-ADP-ribosylation. Collectively, these findings suggest that physiological GSV trafficking depends in part on the presence of IRAP in these vesicles, and that this process is regulated by tankyrase and probably its PARP activity.

Key words: adipocytes, GLUT4, GLUT1, insulin-responsive aminopeptidase (IRAP), iodoxanol gradients, tankyrase.

Abbreviations used: AS160, Akt substrate of 160 kDa; DMEM, Dulbecco’s modified Eagle’s medium; GLUT, glucose transporter; GSK, glycogen synthase kinase; GST, glutathione S-transferase; GSV, glucose transporter 4 storage vesicles; HDM, heavy microsomes; IRAP, insulin-responsive aminopeptidase; IRS-1, insulin receptor substrate-1; LDM, light microsomes; PAR, polymers of ADP-ribose; PARP, poly-ADP-ribose polymerase; PARsylate, poly-ADP-ribosylate; PM, plasma membrane; PNS, post-nuclear supernatant; siRNA, small interfering RNA; TfR, transferrin receptor.

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Golgi region [15,16] and co-localizes with perinuclear GSVs in adipocytes [16]. Its ankyrin-repeat domain contains five IRAP-binding sites [17]. This domain also binds to additional partners such as Grb14 (growth-factor-receptor-bound protein 14) [17,18], a signalling adapter that modulates the glucose-lowering effect of insulin [19]. Most of these partners bind to the ankyrin-repeat domain of tankyrase using an RxPDG sequence motif that corresponds to amino acids 96–101 in the IRAP cytosolic tail [16,17].

Overexpression of this tankyrase-binding region of IRAP, unlike the two aforementioned IRAP fragments, fails to cause insulin-independent GLUT4 translocation [14]. This prompted us to speculate that IRAP binding to tankyrase is not involved in sequestering GSVs but instead might modulate other aspects of GSV trafficking [16].

The functions of tankyrase likely overlap with the closely related tankyrase-2, which oligomerizes with tankyrase and also binds to IRAP [17,20]. Both tankyrases exhibit an unusual catalytic activity known as PARP (poly-ADP-ribose polymerase) activity [20,21], which can modify tankyrases themselves as well as IRAP and other partners through the addition of PAR (polymers of ADP-ribose) [16]. PAR formation (PARylation) is readily reversed through hydrolysis. Circumstantial evidence suggests that cellular PARP activity is involved in glucose homoeostasis. First, nicotinamide, a vitamin that can inhibit PARPs at pharmacological levels, is known to inhibit glucose uptake in cultured adipocytes [22]. Moreover, in knockout mice lacking either the entire tankyrase-2 protein or just the PARP domain, the only obvious phenotype is the reduction in body weight (by up to 20%) and adiposity [23,24], leading to the speculation that tankyrase-2 might regulate energy metabolism [23].

In the present study, we have investigated the role of GLUT4, IRAP and tankyrase in GSV trafficking in 3T3-L1 adipocytes. We found that GSV translocation was impaired by IRAP depletion but not by GLUT4 depletion. Interestingly, depletion of tankyrase or pharmacological inhibition of its PARP activity altered the intracellular distribution of GSVs and also attenuated their insulin-stimulated FM translocation.

**MATERIALS AND METHODS**

**Adipocyte electroporation**

3T3-L1 pre-adipocytes (American Type Culture Collection; Manassas, VA, U.S.A.) were maintained in DMEM (Dulbecco’s modified Eagle’s medium; Cellgro) containing 0.1 % bovine serum albumin (Sigma) or fractionated in iodixanol gradients (see below).

Glucose uptake assays

Adipocytes were seeded in 24-well plates on day 6 and serum-starved on day 8 for 2 h as described in [26]. When indicated, PJ34 (Inotek) and sorbitol (Sigma) were added at 80 µM and 600 mM respectively for 45 min. After insulin stimulation (20 nM for 20 min), [3H]deoxy-D-glucose was added (0.1 µCi at 60 Ci/mmol; MP Biomedicals). After a 10 min incubation at 37°C, cells were rinsed twice with PBS and solubilized in 1 M NaOH (400 µl/well). Aliquots (10 µl) were removed for protein analysis using a kit from BioRad. The remainder was subjected to scintillation counting, and the tracer uptake was normalized to the protein content.

**Immunoadsorption of GLUT1-containing vesicles**

Post-nuclear supernatants harvested as described below from two 10-cm plates of day 8–12 adipocytes were centrifuged at 42,000 g for 75 min. The pellet was resuspended in 1 ml of HES buffer [20 mM Heps (pH 7.5), 0.255 M sucrose and 1 mM EDTA] supplemented with 120 mM NaCl, and preclarified by incubating for 1 h with rabbit immunoglobulin (30 µg) and Protein A–Sepharose (240 µl, CL-4B; Pharmacia) at 4°C. Aliquots (300 µl) of the supernatant were mixed for 1 h with a polyclonal anti-GLUT1 antibody (3 µl, Abcam No. 652, containing 30 µg of immunoglobulin) or control rabbit immunoglobulin (30 µg).

After further incubation with Protein A–Sepharose (80 µl for 2 h), the immune complex was pelleted and washed four times with 120 mM NaCl/HES. The pellet was incubated for 10 min in the same NaCl/HES buffer supplemented with 2 % C12E8 [28], Fluka Biochemical), and the eluates were analysed by SDS/PAGE.

**Differential centrifugation**

Adipocytes electroporated on day 6 were serum-starved on day 8 for 2–3 h in DMEM/0.1 % albumin (Sigma). When indicated, PJ34 (80 µM for 1 h) or insulin (20 nM for 20 min) was added prior to harvesting. Cells were scraped in HES buffer (2.2 ml/10-cm plate) at 4°C and homogenized using a 7 ml Dounce homogenizer (Wheaton). Small aliquots were removed as whole-cell extracts. The remainder was centrifuged at 4°C in an SW60 rotor at 19,000 g for 20 min. From this spin, the pellet was processed as described in [27] to obtain the PM fraction, whereas the PNS (post-nuclear supernatant) was either separated by differential centrifugation into the cytosol fraction as well as LDMs (light microsomes) and HDMS (heavy microsomes) [27], or fractionated in iodixanol gradient (see below).

**Iodixanol equilibrium sedimentation**

The PNS of adipocytes described above was diluted with HES buffer to a protein concentration of 400 µg/ml. Iodixanol [4 g (60 % w/v); Accurate Chemicals] was added to 10.25 g of PBS to obtain a final iodixanol concentration of 14% (w/v). Samples were loaded into OptiSeal tubes (Beckman No. 362181), and density gradients were formed at 57300 rev/min in an NVT65 rotor at 4°C for 4 h. The tubes were then calibrated on the outside, and serial 600 µl fractions were collected from the top for immunoblotting and densitometry analysis. The area under each curve in Figures 6(B) and 7(D) was normalized to 100%. To compare basal with insulin-stimulated samples (Figure 6C),
fractons (45 µl) from both samples were immunoblotted and quantified in parallel.

Affinity precipitation of tankyrase

To concentrate tankyrase-1 and -2 for immunoblotting (Figures 3A and 7A), adipocytes were lysed in buffer A as described in [16] containing 1% Triton X-100 and clarified at 13 000 g for 10 min. Equal protein amounts were incubated with GST (glutathione S-transferase)–IRAP vp-A or -B resins (15 µg, [16]) at 4°C for at least 2 h to pull down both tankyrases.

Immunoblotting and statistical analyses

Primary antibodies were directed against tankyrase (1 µg/ml H-350; Santa Cruz); tankyrase-2 (10 µg/ml, T12; [16]); IRAP (1:12 000 [16]); GLUT4 (5 µg/ml, IF8; [16]); GLUT1 (1:1000, ab652; Abcam); TIR (transferrin receptor; 1:1000; Zymed); caveolin-1 and phospho-tyrosine (both at 1:1000; Transduction Lab); ras (0.5 µg/ml; Transduction Lab); AS160 (1:500; Abcam); poly(ADP-ribose) (1 µg IgY/ml; Tulip Bios labs); IRS-1 (insulin receptor substrate-1; Upstate); IRβ (insulin receptor β; C19; Santa Cruz); actin (1:2000; Sigma); sortilin (1:500; [29]); GSK (glycogen synthase kinase)-3 (Ser7), phospho-Akt S473, and PAS (phospho-specific Akt substrate), the latter three were from Cell Signaling and used at 1:1000. The immunoblots were quantified by densitometry using Kodak 1D image analysis software. The intensity of each band was normalized to the lane representing the control (adipocytes not subjected to knockdown, or insulin or PJ34 treatment) prior to being pooled between experiments for statistical analyses. P values were calculated using the Student’s unpaired two-tailed t test.

RESULTS

IRAP translocation remains intact despite substantial GLUT4 depletion

To explore how GSVP interact with their targeting machinery, we electroporated 3T3-L1 adipocytes with siRNA to knock down GLUT4 and examined the impact on GSV trafficking. The electroporation was performed typically on day 6 of adipogenesis, since more mature adipocytes exhibited a lower viability during the procedure. We found that two independent siRNAs, G4-A and G4-B, caused ~80% GLUT4 depletion in whole-cell extracts harvested on day 8, but had no effect on GLUT1, caveolin-1 or actin (Figure 1A). Interestingly, both G4-A and G4-B as well as a third GLUT4 siRNA (G4-C, results not shown) caused a mild decrease in IRAP expression, a trend that mirrored the IRAP down-regulation in GLUT4 knockout mice [6–8] and presumably reflected IRAP destabilization due to GLUT4 deficiency. As expected, GLUT4 knockdown decreased insulin-stimulated glucose uptake by 35–40% while having little effect on basal glucose uptake (Figure 1B), consistent with GLUT4 mediating only a minor portion of basal uptake [30].

Next, to assess GSV translocation, we immunoblotted the PM fraction for IRAP, an established GSV marker [2]. Despite the tendency to express less IRAP than control, GLUT4-knockdown adipocytes recruited significantly more IRAP to the PM after insulin stimulation (Figure 1C, lanes 2 and 6 compared with lanes 1 and 5). A similar trend has been reflected in fat-specific GLUT4 knockout mice, where adipocytes stimulated ex vivo with insulin recruit more IRAP to the PM than wild-type controls (50% compared with 31% of total IRAP) [8]. Figure 1(C) also shows that GLUT4 knockdown did not impair the PM targeting of GLUT1 or caveolin-1. Thus substantial GLUT4 depletion did not attenuate insulin-stimulated PM translocation of IRAP and, by inference, GSVPs.

IRAP knockdown impairs GLUT4 translocation

In a reciprocal experiment, we explored whether GSV trafficking is dependent on the presence of IRAP. Of the four siRNAs designed against IRAP (also known as vp-165), vp-A and vp-B achieved knockdown most effectively (Figure 2A lanes 2 and 3, and results not shown). Compared with a scrambled siRNA (Scr, lane 4) or buffer alone (buff, lane 1), the glucose uptake after vp-A or vp-B electroporation was normal in the basal state but was reduced in the insulin-stimulated state by ~45% (Figure 2A, graph). A modest reduction of insulin-stimulated glucose uptake was also observed using two other less effective IRAP siRNAs (vp-C and -D, results not shown). Therefore IRAP knockdown attenuated insulin-stimulated glucose uptake in adipocytes. This effect was not due to decreased GLUT4 or GLUT1 expression (Figure 2B).

To explore how IRAP knockdown affected glucose uptake, we purified the PM fraction of adipocytes transfected with vp-A or a scrambled siRNA. Figure 2(C) shows that IRAP knockdown attenuated insulin-stimulated GLUT4 translocation to the PM (lane 3 compared with 4), indicating that the translocation depended in part on the presence of IRAP. This effect was specific, since the PM targeting of the TIR was not affected (lane 3 compared with 4). Unexpectedly, IRAP knockdown did impair GLUT1 translocation to the PM (Figure 2C), an effect that probably contributed to the decreased glucose uptake in these cells (Figure 2A).

A plausible explanation for why IRAP knockdown impaired the translocation of GLUT1 but not TIR would be preferential IRAP–GLUT1 co-localization in vesicular compartments, leading to partially linked trafficking of the two proteins. This notion is consistent with the report that insulin-stimulated increase of GLUT1 in PM (6-fold) approaches the ~8–14-fold increase in GLUT4/IRAP, and exceeds the approx. 3-fold increase in TIR (for references, see [29]). The co-localization of GLUT1 with GLUT4/IRAP is also supported by the reported recovery of 85% of cellular GLUT1 in vesicles immunoabsorbed using anti-GLUT4 antibodies [31]. To directly assess IRAP–GLUT1 co-localization, we immunoabsorbed GLUT1-containing vesicles using an antibody that recognized the GLUT1 N-terminal cytosolic tail. This resulted in substantial depletion of GLUT1 from the input material (Figure 2D, lane 1 compared with lane 2). We were unable to directly demonstrate immunoprecipitated GLUT1 due to interference from the immunoglobulin heavy chain on Western blots. Next, we used the non-ionic detergent C12E8 [28] to elute co-purified proteins from immunoabsorbed GLUT1 vesicles. By comparing the IRAP to TIR ratio between the eluates and the input, Figure 2(D) also shows that GLUT1 vesicles were enriched for IRAP by approx. 2.5-fold over TIR. Thus the substantial presence of IRAP in GLUT1 vesicles might underlie to some extent the impaired GLUT1 translocation following IRAP knockdown.

To show that the decreased translocation of GLUT4 and GLUT1 upon IRAP knockdown was not due to inhibition of insulin signalling, we assessed the signalling using Akt-mediated phosphorylation as a readout. Of particular interest is AS160, an IRAP-binding protein [10] whose phosphorylation by Akt is implicated in GSV translocation [32]. Figure 2(E) shows that IRAP knockdown did not affect insulin-induced gel-mobility shift of AS160 (left-hand panel), nor did it affect insulin-stimulated
Figure 1 GLUT4 knockdown does not impair IRAP translocation

3T3-L1 adipocytes were electroporated on day 6 of differentiation with either a GLUT4 siRNA (G4A or G4B), a scrambled siRNA (Scr) or buffer alone (buf). Cells were insulin-stimulated as indicated (20 nM for 20 min) on day 8 prior to analysis. (A) Whole-cell extracts were immunoblotted (right-hand panel, 30 µg protein/lane) for the indicated proteins. The bar graph to the left shows densitometry quantification (means ± S.E.M.) of the knockdown samples (mean of lanes 2, 4 and 6) normalized against the control (mean of lanes 1, 3 and 5). (B) [3H]Deoxy-D-glucose uptake in the basal state and after insulin stimulation was normalized against the protein content and shown in arbitrary units (a.u.). Each data point represents the means ± S.E.M. of four replicates. (C) The PM fractions were immunoblotted for the indicated proteins (right-hand panel, 15 µg protein/lane), quantified by densitometry, and normalized against the unstimulated control (lane 3). The bar graph to the left shows the mean of insulin-stimulated knockdowns (lanes 2 and 6) and the mean of insulin-stimulated controls (lanes 1 and 5). *P < 0.05 from the control; N.S., not significantly different. Each panel was repeated once (A and C) or twice (B) with similar results. Ctrl, control; KD, knockdown.
phosphorylation of prominent Akt substrates, including a band that co-migrated with AS160, and a 250 kDa band that was presumably the Akt substrate AS250 [33] (right-hand panel). Therefore IRAP knockdown apparently did not affect insulin-Akt signalling.

Tankyrase knockdown attenuates insulin-stimulated glucose uptake

Next, we investigated whether GSV translocation was modulated by the IRAP-binding protein tankyrase [16]. To knockdown tankyrase, we compared eight siRNAs and found T1A and T1B
This phenotypic reversibility suggested that the knockdown by day 9 in T1B-electroporated cells (Figure 3A, lanes 7–9) were immunoblotted for tankyrase (upper panels) and tankyrase-2 (lower panels). The results shown are representative of four (day 8) or two (day 9) experiments. TNKS-1, tankyrase-1; TNKS-2, tankyrase-2.

Figure 3 Tankyrase knockdown impairs glucose uptake reversibly and dose-dependently

Adipocytes were electroporated on day 6 with a tankyrase siRNA (T1A or T1B), a point-mutation control (Pmt) or buffer alone (Buff). (A) Extracts prepared on day 8 (lanes 1–6) or day 9 (lanes 7–9) were immunoblotted for tankyrase (upper panels) and tankyrase-2 (lower panels). (B) [3H]Deoxy-D-glucose uptake in the basal state and after insulin stimulation (20 nM for 20 min) was determined on day 8 (left-hand panel) or day 9 (right-hand panel) as in Figure 1B. The results shown are representative of four (day 8) or two (day 9) experiments. TNKS-1, tankyrase-1; TNKS-2, tankyrase-2.

to be the most effective. We also designed the control siRNA, pmt, by introducing point mutations into T1A. Figure 3(A) shows that in adipocytes electroporated on day 6 and assayed on day 8, T1A achieved >90 % tankyrase depletion (lane 2, upper panel) whereas the control siRNA pmt had no effect (lane 3). The knockdown by T1A was stable through to at least day 9 (results not shown). By comparison, T1B achieved only a modest knockdown on day 8 (lane 5), an effect essentially lost by day 9 (lane 8). Neither T1A nor T1B affected the expression of tankyrase-2 (lower panel). Importantly, insulin-stimulated glucose uptake on day 8 was attenuated robustly by T1A and modestly by T1B (Figure 3B, left-hand panel), indicating a dose-dependent tankyrase effect on glucose uptake.

Tankyrase knockdown conceivably could have attenuated glucose uptake by blocking adipogenic differentiation, since our adipocytes were electroporated on day 6, prior to completing adipogenesis. Arguing against this possibility is that neither T1A nor T1B affected the expression of the adipogenesis markers PPARγ (peroxisome-proliferator-activated receptor γ) and adiponectin (results not shown). Moreover, as tankyrase recovered from the knockdown by day 9 in T1B-electroporated cells (Figure 3A, lane 8), glucose uptake normalized concomitantly (Figure 3B, right-hand panel). This phenotypic reversibility suggested that tankyrase knockdown did not interfere with adipogenesis.

Tankyrase knockdown inhibits GLUT4 and IRAP translocation

To investigate how tankyrase knockdown impaired glucose uptake, we first examined the overall expression of GLUT4 and GLUT1. Figure 4(A) shows that the expression of neither transporter was affected by tankyrase knockdown. Next, we purified the PM fraction of basal and insulin-stimulated adipocytes, and assessed GLUT4 and GLUT1 translocation by immunoblotting. Figure 4(B) shows that tankyrase knockdown impaired the insulin-stimulated PM translocation of GLUT4 (lane 3 compared with 4) but not GLUT1, caveolin-1 or ras. (The translocation of caveolin and ras as previously reported [34,35] was not always discernible in our hands.) As for IRAP, the PM content after insulin stimulation was robustly decreased by tankyrase knockdown (Figure 4B, lane 4 compared with lane 3) while the overall expression was only slightly decreased (Figure 4A), confirming that IRAP translocation was impaired. Therefore tankyrase knockdown specifically impaired the translocation of the major GSV cargo proteins, GLUT4 and IRAP.

Tankyrase knockdown does not affect insulin-induced phosphorylation

A potential mechanism whereby tankyrase knockdown could impair GLUT4/IRAP translocation is through inhibiting insulin signalling, since tankyrase interacts with Gβδ1 [18], an insulin receptor adapter implicated in glucose homeostasis [19], and also with PP1 (protein phosphatase 1) [17], a phosphatase that could conceivably modulate signalling. Arguing against this mechanism is that the knockdown did not prevent insulin signalling from recruiting GLUT1, ras and caveolin-1 to the PM (Figure 4B), nor did the knockdown attenuate insulin-induced phosphorylation of the IR, IRS-1, Akt, GSK3 and p42/p44 ERKs (extracellular-signal-regulated kinases) (Figure 5A, lane 4 compared with lane 2). Moreover, the induction of glucose uptake by osmotic shock, which signals through a non-insulin pathway [5], was also impaired by the knockdown (Figure 5B), supporting the notion that tankyrase knockdown impaired GSV formation or trafficking rather than blocking upstream insulin signalling.

Tankyrase knockdown alters intracellular GLUT4/IRAP distribution

We suspected that the Golgi-associated tankyrase modulated GLUT4/IRAP sorting into exocytosis-competent compartments, rather than directly affecting the exocytosis from these compartments. This is because tankyrase does not move with GSVs to the PM upon insulin stimulation [16] and because vesicular sorting at the Golgi complex and the trans-Golgi network is an integral step of intracellular GSV itinerary [1,36–38]. We therefore hypothesized that tankyrase knockdown altered the basal-state distribution of GLUT4 and IRAP within endosomal compartments. To test this idea, we used differential centrifugation to separate PNSs of serum-starved adipocytes into the cytosolic fraction as well as HDMs and LDMs. We found that tankyrase knockdown apparently did not alter the partitioning of GLUT4 and IRAP among these fractions (Figure 6A); regardless of the knockdown, both proteins were highly enriched in LDMs. (For this reason, a lower percentage of LDMs was loaded in Figure 6A than the other fractions.)

Because the differential centrifugation shown in Figure 6(A) yielded relatively few fractions on the basis of differences in sedimentation rate, it might not detect a subtle redistribution of proteins. We therefore turned to iodixanol (OptiPrep) equilibrium density gradients, which resolved microsomes into 18 fractions on the basis of buoyant density instead of sedimentation rate. This method has been used to separate GLUT4 in LDMs into a denser ‘peak 1’ and a lighter ‘peak 2’, each containing 44 % and 39 % respectively of the input GLUT4 [39,40]. Peak 1 shows a greater depletion of GLUT4 upon insulin stimulation (44 % compared with 25 %), whereas peak 2 is characterized by a higher sortilin content [39]. In the present study, the iodixanol gradients of serum-starved control adipocytes showed a major
Tankyrase and IRAP in GLUT4 exocytosis

Day 6 adipocytes were electroporated with T1A siRNA or a point-mutation control (Pmt). On day 8, (A) whole-cell extracts (30 µg protein/lane) and (B) the PM fractions (15 µg protein/lane) were immunoblotted and quantified as described in Figure 2(C). The bar graph to the left shows the means ± S.E.M. of two basal samples and of four insulin-stimulated samples. *P < 0.05. This experiment was repeated twice with similar results.

GLUT4 peak near the top of the gradient and a minor peak near the bottom (Figure 6B, top panel, dotted curve). The major peak (centred about fraction 5) was sortilin-rich and thus presumably corresponded to peak 2 of previous studies [39,40]. Whether our minor GLUT4 peak (centred about fraction 13) corresponded to peak 1 of previous studies was unclear, since its GLUT4 content was much lower than would have been expected of peak 1. Figure 6(B) also shows that the IRAP distribution in control adipocytes, albeit more even than GLUT4, also peaked at fractions 5 and 13 (middle panel, dotted curve). Importantly, after tankyrase knockdown, both GLUT4 and IRAP were substantially depleted from the lighter peak and shifted toward the middle and the bottom of the gradients (Figure 6B, top two panels, solid curves). These changes were specific, since the GLUT1 profile in the same gradient was largely unaffected (Figure 6B, lower panel). Thus tankyrase knockdown specifically affected the density profile of compartments that harboured GLUT4 and IRAP in the basal state, resulting in an overall increase in the buoyant density of these compartments. This tankyrase effect was highly reproducible in four batches of electroporated cells; however, the profiles themselves were somewhat variable between experiments.

Since tankyrase knockdown shifted GLUT4 and IRAP away from the lighter fractions of iodixanol gradients (Figure 6B) and also impaired their insulin-stimulated translocation (Figure 4B), we suspected that the lighter fractions might contain GLUT4 and IRAP that were highly insulin-responsive and exocytosis-competent. Indeed, when control adipocytes were stimulated with insulin, the greatest depletion of intracellular GLUT4 and IRAP was from the lighter fractions and particularly fraction 5 (Figure 6C, solid curves compared with dotted curves), consistent with these fractions harbouring highly exocytosis-competent GSVs. A caveat is that intracellular redistribution, rather than exocytosis, could also deplete a protein from a given fraction. Nevertheless, our findings are consistent with the notion that altered
basal-state GLUT4/IRAP partitioning within endosomal compartments (Figure 6B) contributed to their impaired translocation upon insulin stimulation (Figure 4B).

**DISCUSSION**

We have investigated the regulation of GLUT4 and IRAP trafficking in 3T3-L1 adipocytes by combining siRNA-mediated knockdown with subcellular fractionation. Our data indicate that substantial depletion of GLUT4 does not affect the insulin-stimulated translocation of IRAP. In contrast, the presence of IRAP is important to insulin-stimulated GLUT4 translocation and glucose uptake. Moreover, the intracellular distribution of GLUT4/IRAP and their insulin-stimulated translocation are regulated by the IRAP-binding protein tankyrase.

In our GLUT4-depleted adipocytes, the intact IRAP translocation (Figure 1C) is in agreement with the robust IRAP translocation reported in tissue-specific GLUT4 knockout mice [7,8] as well as in adipocytes derived from NIH-3T3 cells without inducing GLUT4 expression [43]. These findings collectively suggest that GLUT4 is dispensable for physiological trafficking of IRAP and, by inference, GSVs. Although GLUT4 is known to interact with components of the GSV targeting machinery [44-46], we suspect that GSVs lacking GLUT4 can nevertheless interact with the targeting machinery by utilizing IRAP as a handle.

In IRAP-depleted adipocytes, the impaired insulin-stimulated GLUT4 translocation and glucose uptake (Figures 2A and 2C) raise the possibility that IRAP contributes to the insulin responsiveness of GSVs, whereas GLUT4 may be merely a passenger therein. This notion is supported by direct IRAP interaction with PARsylation machinery [44-46], suggesting that GLUT4 is dispensable for physiological trafficking of IRAP and, by inference, GSVs. Although GLUT4 is known to interact with components of the GSV targeting machinery [44-46], we suspect that GSVs lacking GLUT4 can nevertheless interact with the targeting machinery by utilizing IRAP as a handle.

Given that GSV translocation apparently depended on IRAP (Figure 2C), we investigated whether the translocation was modulated by the IRAP partner tankyrase [16]. We found that upon tankyrase knockdown, the insulin-stimulated translocation of IRAP and GLUT4 as well as glucose uptake were attenuated (Figures 3B and 4B). We therefore speculate that GLUT4/IRAP sorting into exocytosis-competent compartments is promoted by the Golgi-associated tankyrase. This notion is in line with the observations that the Golgi constitutes part of the recycling path of GLUT4/IRAP and that the Golgi-associated coat protein GGA promotes the entry of nascent GLUT4/IRAP into exocytosis-competent compartments [1,36-38]. Although the exact sorting step(s) regulated by tankyrase remains unclear, it might involve GLUT4/IRAP movement between compartments that exhibit different buoyant densities in iodixanol gradients. This would explain why tankyrase depletion apparently traps GLUT4/IRAP in compartments of relatively high buoyant densities (Figure 6B), presumably by blocking their entry into lower-density
Figure 6  Effect of tankyrase knockdown on intracellular GLUT4 and IRAP distribution

(A) PNSs of adipocytes electroporated on day 6 with T1A (lanes 1–3) or the point-mutation control pmt (lanes 4–6) and serum-starved on day 8 were subjected to differential centrifugation. From each 10 cm plate of adipocytes, 1% of the LDM (lanes 1 and 4), 5% of the HDM (lanes 2 and 5) and 1.5% of the cytosolic fraction (lanes 3 and 6) were loaded on to SDS gels for immunoblotting. The blots shown are representative of five independent experiments. (B) Day 6 adipocytes were electroporated with T1A (solid curves) or the control pmt (dotted curves) and harvested on day 8 after a 2 h serum starvation. PNS of controls and knockdowns were resolved in parallel in 14% self-forming iodixanol density gradients, and fractions were immunoblotted for the indicated proteins. The effect of the knockdown shown is representative of four batches of electroporated cells. The insets compare knockdowns (left-hand lane) with controls (right-hand lane) (20 µg of protein/lane) for the amount of GLUT4 and IRAP in the input PNS. (C) Adipocytes electroporated with the control siRNA pmt as in (B) were stimulated with 20 nM insulin for 20 min. PNS were fractionated in 14% iodixanol gradients, immunoblotted for GLUT4 and IRAP in parallel with (B), and plotted as solid curves. The dotted curves representing basal controls were taken from (B). The insulin effect shown is representative of two independent experiments. T1 KD, tankyrase 1 knockdown.
compartments that are highly insulin responsive (Figure 6C). Whether tankyrase modulates GSV trafficking by binding to IRAP remains to be demonstrated. Besides tankyrase, several known regulators of GSV trafficking also bind to IRAP [10–13]. Thus IRAP probably has additional roles in vesicular trafficking that are tankyrase-independent. Supporting this notion is that GLUT1 translocation is impaired by IRAP knockdown (Figure 2C) but not by tankyrase knockdown (Figure 4B).

Since the effects of tankyrase knockdown on intracellular GLUT4/IRAP distribution (Figure 6B) and PM translocation...
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