

## Insulin triggers surface-directed trafficking of sequestered GLUT4 storage vesicles marked by Rab10

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**U**nderstanding how glucose transporter isoform 4 (GLUT4) redistributes to the plasma membrane during insulin stimulation is a major goal of glucose transporter research. GLUT4 molecules normally reside in numerous intracellular compartments, including specialized storage vesicles and early/recycling endosomes. It is unclear how these diverse compartments respond to insulin stimulation to deliver GLUT4 molecules to the plasma membrane. For example, do they fuse with each other first or remain as separate compartments with different trafficking characteristics? Our recent live cell imaging studies are helping to clarify these issues. Using Rab proteins as specific markers to distinguish between storage vesicles and endosomes containing GLUT4, we demonstrate that it is primarily internal GLUT4 storage vesicles (GSVs) marked by Rab10 that approach and fuse at the plasma membrane and GSVs don't interact with endosomes on their way to the plasma membrane. These new findings add strong support to the model that GSV release from intracellular retention plays a major role in supplying GLUT4 molecules onto the PM under insulin stimulation.

Insulin stimulates glucose uptake into adipocytes and muscle tissues by recruiting GLUT4 molecules from intracellular sites to the plasma membrane (PM).<sup>1-3</sup> In the absence of insulin stimulation, the majority of GLUT4 molecules are stored in small intracellular vesicles referred to as GLUT4 storage vesicles (GSVs).<sup>4-6</sup> Following insulin secretion from the pancreas after a meal, insulin receptors on the

surface of muscle cells and adipocytes are engaged by insulin. This sets off a signaling cascade involving PI3K, AKT/PKB, AS160,<sup>7-9</sup> and Rab proteins<sup>10-13</sup> that leads to GLUT4 redistribution from GSVs to the PM. Consequently, levels of GLUT4 molecules at the PM rise by ~30 fold.<sup>14,15</sup>

Understanding the precise membrane trafficking steps that underlie this dramatic buildup of GLUT4 proteins on the PM under insulin stimulation has been challenging. This is because GLUT4 molecules don't only reside in GSVs.<sup>16-18</sup> GLUT4 antibody uptake assays have shown that GLUT4 proteins continuously recycle through early and recycling endosomes.<sup>19</sup> Because GLUT4 resides in both endosomes and GSVs, the pathway by which GLUT4 molecules redistribute from GSVs to the PM during insulin stimulation could be direct or indirect. That is, GLUT4 proteins could be delivered to the PM by direct fusion of GSVs with the PM, or by an indirect pathway involving initial fusion of GSVs with endosomes followed by later fusion of endosomes with the PM.<sup>2,20</sup>

Previous live cell imaging experiments attempting to distinguish between these models employed total internal reflection (TIRF) microscopy and a GLUT4-EGFP probe to visualize vesicles in close proximity to the PM. Hundreds of GLUT4-GFP-containing vesicles close to the PM were observed in both insulin-stimulated and non-stimulated cells.<sup>21-24</sup> Indeed, the number of GLUT4-GFP vesicles visualized did not change before or during insulin treatment.<sup>21</sup> Whenever a fraction of GLUT4-GFP vesicles fused with the PM, more vesicles moved into the TIRF zone

**Keywords:** GLUT4, IRAP, Rab10, Rab14, AS160, adipocytes, insulin, TIRF

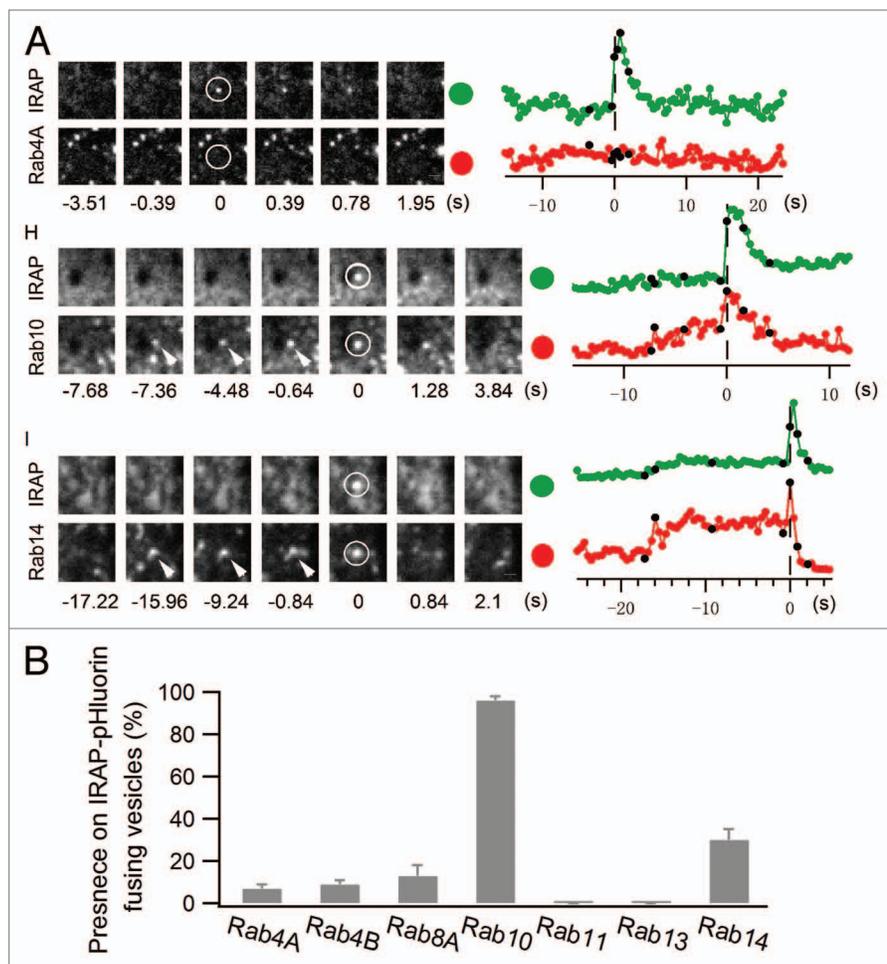
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Submitted: 08/08/2013

Revised: 09/10/2013

Accepted: 09/12/2013

<http://dx.doi.org/10.4161/sgtp.26471>



**Figure 1.** Rab10 and Rab14 label exocytic GLUT4 vesicles. Rab proteins tagged with TagRFP were separately transfected into adipocytes along with IRAP-pHluorin. (A) IRAP-pHluorin fusion events were monitored using dual-color TIRF microscopy 3 min after insulin stimulation for the presence of a particular Rab protein on the fusing vesicles. Fusion site intensities were measured from both channels and plotted to the right. Black dots on the intensity traces indicate the time points at which image frames to the left are extracted. Scale bar: 0.5  $\mu$ m. (B) Summary of the presence of different Rab proteins on IRAP-pHluorin fusing vesicles.

to effectively replace them. Because the sizes of all the vesicles were below the diffraction limit of fluorescence microscopy, it was difficult to determine whether any vesicle that fused with the PM was a GSV or endosomal vesicle.<sup>25,26</sup> Without probes to discriminate GSVs from endosomal compartments, therefore, addressing whether insulin-stimulated GLUT4 redistribution to the PM occurs by a direct or indirect route is unfeasible.

One group of markers capable of distinguishing GSVs from endosomes is the set of Rab proteins. These small GTPases function to modulate the surface characteristics of different subcellular organelles and help to define organelle identity.<sup>27,28</sup> By determining which Rab proteins

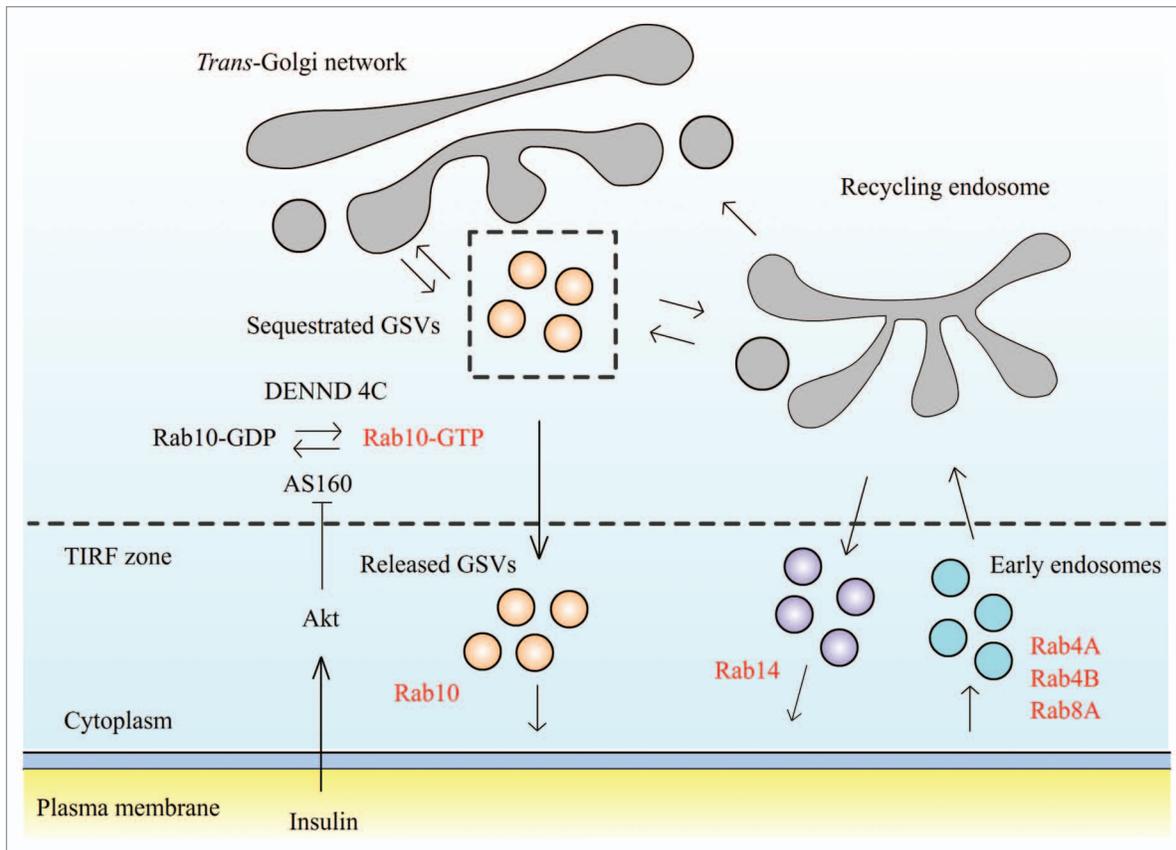
associate with GSVs and which with GLUT4-positive endosomal compartments, we reasoned it should be possible to distinguish between GSVs and endosomes in TIRF imaging experiments, and thereby address whether insulin-induced arrival of GLUT4 at the PM occurs by a direct or indirect route.

Toward this goal, 25 candidate Rab proteins were screened for their co-localization with GLUT4-containing vesicles close to the PM and their ability to fuse with the PM during insulin stimulation.<sup>29</sup> To monitor GLUT4 vesicle fusion with the PM, we expressed the insulin responsive aminopeptidase (IRAP, which always co-localizes with GLUT4) tagged with pHluorin (IRAP-pHluorin).<sup>30</sup> Because

pHluorin produces a bright flash of light when it shifts from acidic to neutral pH,<sup>31</sup> acidic intracellular vesicles containing IRAP-pHluorin could be visualized as they fused at the PM and became exposed to neutral pH. Screening 25 Rab protein family members using IRAP-pHluorin, we found that both Rab 10 and 14 were associated with IRAP-pHluorin vesicles that underwent fusion at the PM in response to insulin treatment (Fig. 1). Moreover, Rab10 vesicles showed little overlap with Rab14 vesicles and vice versa, suggesting each Rab protein was associated with a different subcellular compartment.<sup>29</sup>

We next addressed whether Rab10 and Rab14 vesicles were positive for transferrin receptor (TfR, a receptor that constitutively cycles through the endosomal system). Nearly all Rab14 vesicles contained TfR, whereas virtually no Rab10-containing vesicles had TfR. These results indicated that Rab14 marked GLUT4-containing endosomes, while Rab10 marked GSVs.<sup>32</sup> Because most of the GLUT4-containing vesicles that fused with the PM during insulin stimulation were Rab10 positive, we concluded that GSVs (marked by Rab10) were the primary vehicle by which GLUT4 was delivered to the PM during insulin stimulation. Moreover, since Rab10 vesicles did not fuse with other vesicles prior to PM fusion, the direct trafficking model for insulin-stimulated GLUT4 redistribution to the cell surface was supported.

To verify the role of Rab10 and Rab14 in delivery of GLUT4 to the PM under insulin stimulation, we knocked them down using siRNA technology. Rab10 knockdown significantly reduced insulin-stimulated GLUT4 translocation to the PM. This further confirmed that Rab10 activity on GSVs facilitates direct trafficking of GSVs to and fusion with the PM during insulin stimulation. Rab14 knockdown, by contrast, only modestly inhibited GLUT4 translocation. This provided evidence against the indirect model involving merging of GSVs and endosomes followed by endosome-PM fusion since Rab14 knockdown would be expected to have a major inhibitory effect in this scenario. When both Rab10 and Rab14 were knocked down, we observed



**Figure 2.** Schematic model of GLUT4 trafficking in adipocytes. Insulin stimulation inhibits AS160 GAP activity, resulting in Rab10 shifting to GTP-bound active state. Rab10 activation releases intracellularly retained GLUT4 storage vesicles (GSVs) to the cell periphery, where they dock and fuse at the cell membrane. Meanwhile, insulin stimulation promotes Rab14-associated endosomal compartments to fuse at the cell membrane, which functions as a parallel pathway to deliver GLUT4 to the PM. At the cell membrane, GLUT4 molecules are endocytosed and travel through a variety of endosomal compartments labeled by multiple Rab proteins before being reloaded into GSVs.

an additive inhibitory effect on GLUT4 translocation. This raised the possibility that GSV-PM fusion (facilitated by Rab10) and endosome-PM fusion (facilitated by Rab14) independently contribute to insulin-stimulated delivery of GLUT4 to the PM.<sup>29</sup>

Identification of Rab10 as a GSV marker and Rab14 as a GLUT4-containing endosome marker has been a critical step in understanding insulin-stimulated trafficking pathway(s) for GLUT4 translocation to the PM. Previously, proteins used to study GLUT4 trafficking were either other cargo molecules that shared an identical trafficking itinerary with GLUT4 (e.g., IRAP)<sup>33</sup> or didn't solely mark GSVs (e.g., VAMP2 and sortilin).<sup>34,35</sup> Given our finding that Rab10 activity is necessary for delivering GSVs to the PM after insulin stimulation, Rab10 would appear to be a unique marker among all other GLUT4

compartmental probes for highlighting GSVs poised to fuse with the PM.

Using Rab10 as a specific GSV marker, we explored in detail the identities and activities of GLUT4-containing compartments in the TIRF zone. In the absence of insulin stimulation, Rab10-labeled GSVs were barely observable in the TIRF zone. This suggested that GSVs are sequestered internally, away from the cell periphery, in the basal state. Rab4A and Rab4B, 2 endocytic Rab proteins,<sup>28</sup> co-localized extensively with GLUT4 in the TIRF zone under these conditions. This implied GLUT4 constitutively recycles through endocytic structures close to the PM in the basal state. After insulin stimulation, Rab10-labeled GSVs entered into the TIRF zone and fused at the PM. Fusion of Rab10-labeled GSVs at the PM was very efficient once GSVs moved into the TIRF zone. Indeed, GSV dwell time in the

TIRF zone was extremely short compared with that of endosomal GLUT4-containing compartments. This helps explain why GSVs comprise only a small fraction of the total GLUT4 compartments in the TIRF zone both before and after insulin stimulation, and why GLUT4 vesicle density in the TIRF zone barely changes under these conditions.

While the majority of the GLUT4 molecules arriving at the PM under insulin stimulation did so as a result of GSV-PM fusion events, our data also suggested a contribution from endosome-PM fusion events, in particular, PM fusion of Rab14-positive, GLUT4-containing endosomal vesicles. This pathway might be important to maintain GLUT4 at the PM after GSV stores of GLUT4 are depleted during insulin stimulation,<sup>25</sup> in addition to recycling GLUT4 back into GSVs as proposed by other groups.<sup>36,37</sup> By being able

to access GLUT4 molecules that recycle through endosomes, the cell would be able to maintain its GLUT4 PM pool during insulin stimulation even without GSVs.<sup>25</sup>

The above results suggest GLUT4 proteins during insulin stimulation are primarily delivered to the PM by direct fusion of GSVs with the PM. Rab14-containing endosomal vesicles also help redistribute GLUT4 onto the PM but the major pathway is through direct GSV-PM fusion. What seems to occur is that Rab10-containing GSVs are released from intracellular sequestration upon insulin stimulation and this enables them to move in a directed fashion to the cell periphery.<sup>29</sup> The emerging consensus in favor of this direct trafficking model<sup>20</sup> has led to interest in its molecular mechanisms (Fig. 2). The Rab10 GAP protein, AS160, has been suggested to negatively regulate GSV mobilization. In this view, AS160 functions in the absence of insulin stimulation as an active GAP causing Rab10 to be in its GDP-binding inactive state.<sup>8,38</sup> Upon insulin stimulation, AS160 phosphorylation by Akt turns off AS160's GAP domain, allowing GEF Dennd4c to convert Rab10 to its GTP-binding active state.<sup>10,39</sup> Rab10 in the active state attaches to GSVs and recruits downstream effectors, including kinesin<sup>40,41</sup> and myosin motors,<sup>14,42</sup> to physically translocate GSVs close to the PM. This scenario is supported by our observations that Rab10 is already associated with GSVs before they move into the TIRF zone and that a constitutively active Rab10 mutant induces GLUT4 accumulation at the PM in the absence of insulin stimulation.<sup>29</sup> An additional GSV sequestration mechanism may involve TUG (a putative tethering factor for GLUT4 that contains a ubiquitin-like UBX domain).<sup>43-45</sup> TUG has been shown to directly interact with GLUT4 molecules and could retain GSVs deep in the cell in the absence of insulin.<sup>43</sup> When TUG is cleaved upon insulin stimulation, GSVs are released and become ready to be mobilized.<sup>45</sup> Activated Rab10 then attaches to the GSVs and facilitates their delivery to the PM.

In conclusion, live cell imaging studies using different Rab proteins have been successful at dissecting the complex trafficking routes followed by GLUT4 molecules

under insulin stimulation. What is now needed is a better understanding of the specific roles these Rab proteins play in controlling GLUT4 trafficking. It is clear that Rab10 is necessary for GSV translocation to the PM, but is this GTPase also involved in GSV biogenesis and/or GSV fusion with the PM? Likewise, because Rab4 predominantly localizes at steady-state with GLUT4 vesicle adjacent to the PM, could its function be to retrieve GLUT4 off the PM and/or to help store GLUT4? These and other questions related to Rabs and GLUT4 trafficking now are ripe for addressing.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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