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Grp1 plays a key role in linking insulin signaling to glut4 recycling

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SUMMARY

The glucose transporter type 4 (glut4) is critical for metabolic homeostasis. Insulin regulates glut4 by modulating its expression on the cell surface. This regulation is achieved mainly by targeting the endocytic recycling of glut4. We identify Grp1 (general receptor for 3-phosphoinositides 1) as a GEF (guanine nucleotide exchange factor) for ARF6 (ADP-ribosylation factor 6) that promotes glut4 vesicle formation. Grp1 also promotes the later steps of glut4 recycling through ARF6. Insulin signaling regulates Grp1 through phosphorylation by Akt. We also find that mutations which mimic constitutive phosphorylation of Grp1 can bypass upstream insulin signaling to induce glut4 recycling. Thus, we have uncovered a major mechanism by which insulin regulates glut4 recycling. Our findings also reveal the complexity by which a single small GTPase in vesicular transport can coordinate its multiple steps to accomplish a round of transport.

INTRODUCTION

The glucose transporter type 4 (glut4) is expressed selectively in fat and muscle tissues, where it plays a direct role in glucose homeostasis, and also has indirect roles in other metabolic events such as fatty acid biogenesis (Herman and Kahn, 2006; Huang and Czech, 2007; Petersen and Shulman, 2006). Insulin regulates glut4 by modulating its surface expression, which is achieved mainly by targeting the endocytic recycling of glut4 (Bogan and Kandror, 2010; Foley et al., 2011; Huang and Czech, 2007; Rowland et al., 2011; Watson and Pessin, 2006). The understanding of how upstream insulin signaling affects the downstream process of glut4 recycling is predicted to shed molecular insights into major metabolic disorders, such as type 2 diabetes mellitus. Moreover, this elucidation contributes

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to a basic understanding of regulated transport, as glut4 recycling has been a key example of how intracellular signaling can act in complex ways to affect vesicular transport.

Insulin binding to its receptor results in the recruitment of downstream signaling components that include insulin receptor substrate (IRS), phosphatidylinositol 3-kinase (PI3K), and the protein kinase Akt (Huang and Czech, 2007; Watson and Pessin, 2006). Akt is considered a key distal component of insulin signaling, as it often acts at the nexus that links insulin signaling with its downstream events, including glut4 recycling (Ng et al., 2008). The identification of key transport factors that act in glut4 recycling has been facilitated by the general paradigm that vesicular transport involves a series of highly conserved mechanistic steps that are performed by different families of core effectors. Clathrin that couples with a recently defined adaptor, known as ACAP1 (Arfgap with Coil-coil and Ankyrin repeats Protein 1), has been identified to act as a coat complex that initiates glut4 recycling from early endosomes (Li et al., 2007). Myo1c has been identified to act in the translocation of glut4 vesicles to the plasma membrane (PM) (Bose et al., 2002; Chen et al., 2007; Yip et al., 2008). The exocyst has been identified to act in the docking of glut4 vesicles to the PM (Chen et al., 2007; Inoue et al., 2003). Specific SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complexes have also been identified to act in the fusion of glut4 vesicles to the PM (Cheatham et al., 1996; Martin et al., 1996; Williams and Pessin, 2008).

Small GTPases act as key regulators of cellular events (D'Souza-Schorey and Chavrier, 2006). ARF6 has been identified to regulate the clathrin ACAP1-containing coat complex for the initial step of glut4 recycling (Li et al., 2007). RalA and Rab10 have been identified to regulate motor proteins and/or the tether complex for the later steps of this recycling (Chen et al., 2007; Sano et al., 2007). Small GTPases cycle between active (GTP-bound) and inactive (GDP-bound) states, which require guanine nucleotide factors (GEFs) to catalyze activation and GTPase-activating proteins (GAPs) to catalyze deactivation (Bos et al., 2007). The GAPs for RalA and Rab10, known as RalA GAP complex (Chen et al., 2011) and AS160 (Eguez et al., 2005; Sano et al., 2003) respectively, have been identified as targets of upstream insulin signaling. Other transport factors that are also targeted by insulin signaling include Munc18 (Jewell et al., 2011), Myo1c (Yip et al., 2008), Synip (Min et al., 1999; Yamada et al., 2005), and TUG (Bogan et al., 2003; Xu et al., 2011).

Notably, all these transport factors that are currently known to be targeted by upstream insulin signaling act in the translocation, docking or fusion of glut4 vesicles with the PM. As such, this circumstance has also contributed to the current view that insulin promotes glut4 recycling by targeting mainly its later steps (Foley et al., 2011; Huang and Czech, 2007; Rowland et al., 2011; Watson and Pessin, 2006). This view is seemingly further supported by the observation that glut4 vesicles are detected in the basal (no insulin) condition (Slot et al., 1991), suggesting that glut4 vesicle formation occurs without insulin stimulation. In recent years, results from live-imaging studies that have focused on the behavior of glut4 vesicles near the PM seem to provide further support to the current view, as these studies have directly observed the regulation of glut4 vesicle docking and/or fusion by insulin (Bai et al., 2007; Koumanov et al., 2005; Lizunov et al., 2005; Stenkula et al., 2010). However, because glut4 vesicle formation has been far less studied, this situation also prevents a complete validation of the current paradigm.

A hallmark of type 2 diabetes is insulin resistance, which often involves defects in insulin signaling. Thus, there has been great interest in identifying transport factors that can bypass defect in upstream insulin signaling to promote glut4 recycling, as such factors have the prospect of correcting a major manifestation of insulin resistance, the reduction in glucose uptake in fat and muscle tissues that leads to hyperglycemia. Among the currently known

targets of insulin, only perturbation of AS160 has been shown to have this capability (Eguez et al., 2005). However, quantitative analysis shows that this effect by targeting AS160 is relatively modest compared to the effect of insulin stimulation (Eguez et al., 2005), suggesting that additional mechanisms, yet to be defined, are targeted by insulin in promoting glut4 recycling. We now identify Grp1 as an ARF GEF that initiates glut4 vesicle formation. Characterizing this role, we uncover a major mechanism by which insulin regulates glut4 recycling, which also reveals surprising complexity by which the different steps of glut4 recycling can be coordinated to achieve a round of vesicular transport. We also find that the targeted activation of Grp1 can bypass upstream insulin signaling to induce a robust level of glut4 recycling.

RESULTS

Grp1 acts as an ARF GEF to initiate glut4 vesicle formation

ARF GEFs have been classified functionally into two broad categories based on their sensitivity to pharmacologic inhibition by brefeldin-A (BFA) (Casanova, 2007; D'Souza-Schorey and Chavrier, 2006). Glut4 recycling has been shown previously to be insensitive to BFA (Martin et al., 2000). More recently, another compound (known as SecinH3) has been identified that targets a family of BFA-insensitive GEFs, known as the cytohesins (Hafner et al., 2006). Thus, to determine whether a member of this family acts in glut4 recycling, we initially examined the effect of adding SecinH3 to differentiated 3T3-L1 adipocyte, which has been the model cell type for physiologic studies on glut4 recycling. A quantitative microscopy-based approach has been widely adopted in recent years as a more precise way of analyzing glut4 recycling, which involves measuring the level of surface glut4 and normalizing to the total level of glut4 (Chen et al., 2007; Eguez et al., 2005; Li et al., 2007; Ng et al., 2008; Sano et al., 2007; Williams and Pessin, 2008). Performing this glut4 recycling assay, we found that the addition of SecinH3 inhibited insulin-stimulated glut4 recycling in adipocytes (Fig 1A). As confirmation, we also performed the more traditional glucose uptake assay in adipocytes. SecinH3 also showed inhibition in this assay (Fig 1B). Thus, these initial results suggested that a member of the cytohesin family acts in glut4 recycling.

However, we also considered that glut4 recycling is regulated by insulin signaling, and members of the cytohesin family has been shown in recent years to have an additional function as early components in this signaling (Hafner et al., 2006). As this role was first discovered in studying hepatocytes (Hafner et al., 2006), we examined whether the cytohesins would have a similar role in adipocytes. Initially, examining tyrosine phosphorylation in whole cell lysates, we found that SecinH3 did not induce global changes (Fig S1A). We next interrogated more specific readouts of insulin signaling. SecinH3 has been observed previously to inhibit the ability of insulin to activate IRS1 in hepatocytes (Hafner et al., 2006). However, we found that SecinH3 did not have a similar effect on IRS1 in adipocytes, as reflected by phosphorylation at tyrosine residue 612 (Y612) of IRS1 (Fig S1B). Moreover, whereas SecinH3 was observed previously to inhibit the ability of insulin to activate a distal signaling component, Akt (Hafner et al., 2006), we found that SecinH3 also did not have a similar effect on Akt in adipocytes, as reflected by phosphorylation at either threonine residue 308 (T308) or serine residue 473 (S473) of Akt (Fig S1C). We further noted that the depletion of ARF6 by small interfering ribonucleic acid (siRNA) had been shown previously to inhibit insulin signaling in hepatocytes, and thereby also implicating ARF6 to act as an early signaling component (Hafner et al., 2006). However, we found that siRNA against ARF6 (Fig S1D) did not induce a global change in the phosphotyrosine profile of whole cell lysates (Fig S1E). Moreover, knocking down ARF6 did not have an appreciable effect on the activation of IRS1 (Fig S1F) or Akt (Fig S1G). Taken

together, these results revealed that a cytohesin member predicted to act in glut4 recycling was unlikely to function as an early component of insulin signaling.

The cytohesin family is predicted to have four members (Casanova, 2007; D'Souza-Schorey and Chavrier, 2006). As cytohesin-4 is expressed predominantly in hematopoietic cells (Casanova, 2007; D'Souza-Schorey and Chavrier, 2006), we focused on cytohesin-1, cytohesin-2 (also known as ARNO) and cytohesin-3 (also known as Grp1) as candidates. To determine which member acts in glut4 recycling, we sought to target their function by siRNA. We first documented the specificity of each targeting siRNA (Fig 1C). Subsequently, examining glut4 recycling using the quantitative in vivo assay, we found that only siRNA against Grp1 led to appreciable inhibition in glut4 recycling (Fig 1D). Moreover, consistent with our conclusion above that a potential role for a cytohesin member in glut4 recycling was unlikely to involve its role in insulin signaling, we found that siRNA against Grp1 did not have an appreciable effect on the ability of insulin to activate Akt (Fig 1E).

We next sought further confirmation for the specificity of the targeting siRNA, as a siRNA-resistant form of wild-type Grp1 could rescue the inhibition of glut4 recycling induced by the oligonucleotides used for siRNA against Grp1 (Fig 1F). We also found that a catalytic-dead point mutation in Grp1 (E161K) prevented this rescue (Fig 1F), indicating that the catalytic activity of Grp1 is needed for its role in glut4 recycling. Characterizing the effect of this mutation further, we found that it localized to the glut4-positive endosomal compartment similarly as the wild-type form (Fig S1H). Thus, we concluded that the mutant Grp1 could act in a dominant negative manner to inhibit glut4 recycling, by localizing properly but not being able to activate ARF6 at the target site.

For the above results, we generated stable cell lines to express transfected forms of Grp1, because transient transfection of ARF GEFs that leads to their overexpression has been known to hyper-activate their activity in vivo (Santy and Casanova, 2001). Thus, to avoid the possibility that this enhanced GEF activity might convert glut4 recycling to a constitutive process (instead of being regulated by insulin), we sought to express modest levels of the rescue constructs through stable transfection using a lentiviral expression system. Moreover, because this approach resulted in levels of transfected Grp1 less than that of the endogenous form (Fig 1G), we also sought to reduce the level of endogenous Grp1 by siRNA, so that the potential effects to the transfected forms could be more readily manifested. Consequently, the expression of transfected wild-type form of Grp1 only supported a 5-fold induction in the surface expression of glut4 upon insulin stimulation, as compared to a 9-fold increase in control cells that expressed the higher level of endogenous Grp1 (see Fig 1F). We also ascertained that the use of the lentiviral expression system did not appreciably impact on the ability of the targeting siRNA to deplete endogenous Grp1 (Fig S1J).

We next examined the distribution of Grp1 in adipocytes. By confocal microscopy, we detected a pool of Grp1 that colocalized with internal glut4 in unstimulated cells (Fig 2A). We also compared the distribution of Grp1 with that of ACAP1 and clathrin, which has been shown to act as the coat complex in glut4 recycling (Li et al., 2007), and also that of ARF6, which has been shown to recruit this coat complex to the glut4-positive endosomal compartment (Li et al., 2007). We could detect a pool of Grp1 colocalizing with ARF6, ACAP1 and clathrin heavy chain (CHC) (Fig 2A). Because the GEF acts to recruit the cognate ARF and coat complex to initiate vesicular transport (Casanova, 2007; D'Souza-Schorey and Chavrier, 2006), we next examined whether the depletion of Grp1 by siRNA would reduce the localization of ARF6, ACAP1, and CHC to the internal glut4-positive compartments. We found that ARF6, ACAP1, and CHC all showed decreased localization to

this compartment (Fig S2A), which was further confirmed by quantitation (Fig 2B). The decreased localization could not be attributed to the affected proteins having been degraded, as the protein levels of ARF6, ACAP1, and CHC were not altered by the siRNA treatment (Fig S2B). Further confirming that Grp1 acted upstream to ARF6 and the coat components, we also found that targeting the downstream factors by siRNA did not affect the localization of Grp1 to the internal glut4 compartment (Fig S2C), which was also confirmed by quantitation (Fig 2C). Consistent with these results, siRNA against either ACAP1 or CHC also did not affect the ability of Grp1 to associate with ARF6 (Fig 2D). Thus, the observations altogether led us to conclude that Grp1 acted upstream of ARF6, ACAP1, and clathrin in its role in glut4 recycling.

As these factors are predicted to be involved in vesicle formation, we next sought confirmation by pursuing a previously established glut4 vesicle reconstitution system (Xu and Kandror, 2002). Briefly, fractions that contained either cytosol or compartmental membrane were collected from adipocytes in the basal condition, which ensured that glut4 would reside mostly at internal endosomes rather than at the PM. The two fractions were then incubated in the presence of an ATP-regenerating system. Glut4 vesicle formation was reflected by the redistribution of the transmembrane glut4 from the pellet fraction (which contained the larger compartmental membranes) to the supernatant fraction (which contained the smaller vesicular membranes). When membrane and cytosol fractions were collected from cells that had been treated with siRNA against Grp1 for incubation in the reconstitution system, we found that the redistribution of glut4 was inhibited (Fig 2E). Further validating this reconstitution system, we found that the redistribution of glut4 was also inhibited when membrane and cytosol fractions were collected from cells that had been treated with siRNA against ARF6, ACAP1, or CHC (Fig 2E). Thus, these results supported the conclusion that Grp1 acts as the ARF GEF to initiate glut4 vesicle formation.

Insulin signaling regulates Grp1 by phosphorylating key residues

We next explored whether upstream insulin signaling regulates this role of Grp1. As Akt has been predicted to be a key distal component of the insulin signaling cascade that interfaces with downstream transport factors of glut4 recycling (Ng et al., 2008), we initially pursued an algorithm that predicted residues on substrates targeted by different kinases (Yaffe et al., 2001). This prediction algorithm suggested three serine/threonine residues in Grp1 as potential targets of Akt – serine 155 (S155), threonine 220 (T220), and threonine 280 (T280) (Fig S3A). To determine whether any of these sites regulated the role of Grp1 in glut4 recycling, we first examined whether mutating each residue to alanine (which abrogates potential phosphorylation at the site) would inhibit the ability of insulin to induce glut4 recycling. Technically, we again sought to express modest levels of transfected Grp1 by generating stable cell lines (see Fig 1G), and also reducing the level of endogenous Grp1 through siRNA to allow the effect of the mutant forms to be manifested more clearly. Subsequently, assessing the effects of mutation at each of the three residues, we found that mutations at either S155 (S155A) or T280 (T280A) reduced the ability of insulin to induce glut4 recycling, and as control, mutation at T220 (T220A) did not have a similar effect (Fig 3A).

We next considered the intriguing possibility that mutations to mimic constitutive phosphorylation at the two key residues (S155 and T280) in Grp1 may convert glut4 recycling from a regulated (insulin-dependent) to a constitutive (insulin-independent) process. Indeed, when both residues were mutated to aspartates (S155D and T280D), we found that glut4 recycling occurred despite no insulin having been added, and as control, a similar mutation at T220 (T220D) did not stimulate glut4 recycling (Fig 3B). We also noted that a positive feedback loop has been suggested recently in describing how the cytohesin members are regulated (Stalder et al., 2011). As this type of regulation is predicted to

magnify the effect of the phosphorylation at key residues, we next sought confirmation by mutating only one residue to mimic constitutive phosphorylation (S155D or T280D), and then examining for effects on glut4 recycling in the basal condition. Indeed, either mutation was able to induce glut4 recycling nearly to the same extent as that seen for the “double D” mutation (Fig 3B).

We next sought confirmation that the two key residues affected the activation of ARF6 in vivo. First however, we noted that Grp1 has been suggested to be a more potent activator of ARF1 than ARF6, based on the in vitro assessment of GEF activity (Klarlund et al., 1998), but in vivo studies also suggest that Grp1 acts on ARF6 (Langille et al., 1999). Thus, to resolve this issue more definitely with respect to glut4 recycling, we examined the effect of siRNA against ARF1, and found that it did not affect glut4 recycling (Fig S3B). In contrast, we noted that siRNA against ARF6 had been shown previously to inhibit glut4 recycling (Li et al., 2007). Thus, we next focused on determining whether the two key residues in Grp1 regulated its ability to activate ARF6 in vivo. A general approach has been to use an effector domain to detect the binding of the target small GTPase in its active form, as exemplified by the previous use of a GGA effector domain to detect the activated form of ARF6 in vivo (Santy and Casanova, 2001). Pursuing this approach, we initially could not detect a significant change in ARF6 activation in cells that expressed the different mutant forms of Grp1 (Fig 3C). We next considered that a component of the coat complex involved in glut4 vesicle formation is ACAP1, which also acts as the GAP to deactivate ARF6. As such, ARF6 activation is predicted to be followed by its rapid deactivation during glut4 vesicle formation. As this situation would thwart the ability to detect any significant accumulation of activated ARF6 in vivo, we sought to overcome this hurdle by treating adipocytes with siRNA against ACAP1, and the examining for the effect of stimulating Grp1. In this circumstance, ARF6 activation could be detected when cells were stimulated with insulin, or transfected with the activating mutations of Grp1 (Fig 3C).

We also sought confirmation that the two key residues in Grp1 regulated its role in glut4 vesicle formation by re-visiting the glut4 vesicle reconstitution system. Membrane and cytosol fractions were again collected from adipocytes in the basal condition that had been treated with siRNA against Grp1. The recombinant forms of different mutant Grp1 were then added to the reconstitution system. We found that S155D and T280D promoted glut4 vesicle formation, while S155A and T280A had markedly less effect (Fig 3D). We also sought in vivo confirmation for this result. A subcellular fractionation approach has been used previously to track glut4 vesicle formation from compartmental membrane of endosomes. Briefly, this involves fractionating cytoplasmic membranes by velocity sedimentation to segregate large (compartmental) membrane from small (vesicular) membrane, followed by immunoblotting for glut4 (Li et al., 2007). By this fractionation approach, we found that glut4 resided mostly in the vesicular fraction (Fig 3E). Notably, upon inhibiting Grp1 by siRNA or the expression of the alanine mutants of Grp1, we found that glut4 was redistributed to the compartmental membrane fraction (Fig 3E). Confirming that this redistribution likely represented inhibition at the step of glut4 vesicle formation, we found that siRNA against Myo1c or Sec10, which acted in the translocation and docking steps of glut4 recycling respectively (Bose et al., 2002; Chen et al., 2007; Inoue et al., 2003; Yip et al., 2008), did not induce a similar redistribution (Fig 3E). We further noted that siRNA against ARF6, ACAP1, or CHC had been shown to induce a similar redistribution of glut4 using this fraction approach (Li et al., 2007). Thus, when taken altogether, the observations further supported a key role for Grp1 in glut4 vesicle formation, and also S155 and T280 in regulating Grp1 in this process.

We next examined the distribution of glut4 among intracellular compartments upon the inhibition of Grp1. In control cells, we found by confocal microscopy that glut4 showed

little colocalization with TGN38 [which marked the trans-Golgi network (TGN)], and Lamp1 (which marked the late endosome and lysosomes), while having modest colocalization with transferrin receptor (TfR) (which marked the recycling endosome) (Fig S3C). However, in cells treated with siRNA against Grp1, glut4 showed enhanced colocalization with TfR, but not with the other organellar markers (Figs S3C). These results were also quantified (Fig 3F). We also confirmed that the lack of colocalization between glut4 and Lamp1 was not due to a population of glut4 having been degraded at the lysosome, as immunoblotting of whole cell lysates did not reveal a decrease in the protein level of glut4 when Grp1 was inhibited (Fig S3D). Thus, these results were consistent with a current view that glut4 vesicles are derived from the recycling endosome (Foley et al., 2011).

We next sought confirmation that Akt acted on these two residues in Grp1. Initially, we took advantage of an antibody that detects sites on proteins that are phosphorylated by Akt (Manning et al., 2002). We found that this phospho-Akt substrate antibody showed increased detection of Grp1, when it was isolated from insulin-stimulated cells (Fig 4A). Supporting this finding, we also found that inhibition of Akt, through siRNA, or targeting phosphatidylinositol-3 (PI3) kinase or Akt by pharmacologic inhibition, resulted in reduced detection of Grp1 by this antibody (Fig 4B). The specificity of these inhibitions was reflected by effects on the phosphorylation of Akt and ribosomal protein S6 (S6P) (Manning and Cantley, 2007) (Fig S4A). We also sought confirmation that Akt could act directly on Grp1 by performing an in vitro kinase assay. Upon incubation of purified activated Akt with recombinant Grp1, we detected phosphorylation of Grp1 using the phospho-Akt substrate antibody (Fig 4C). We also found that this phosphorylation could only be abolished by mutating both residues in Grp1, S155 and T280, to alanines (Fig 4C). Thus, these results suggest that we have identified at least two residues in Grp1, S155 and T280, which can be direct targets of Akt.

We next sought insight into how the two residues in Grp1 affected its function. Grp1 has two main functional domains, a Sec7 domain that catalyzes ARF activation and a PH domain that binds to target membrane (see Fig S3A). First, to assess the GEF activity of Grp1, we performed an in vitro GEF assay, which involved incubating recombinant forms of different point mutants of Grp1 with recombinant ARF6. We found that S155D showed enhanced ability to activate ARF6, but the other point mutations did not have a similar effect (Fig 4D and Fig S4B). Second, to assess the recruitment of Grp1 to target membrane, we isolated a membrane fraction from adipocytes that was enriched for internal glut4 (Fig S4C). Upon incubation of this membrane fraction with different point mutants of Grp1 as recombinant proteins, we found that only T280D showed enhanced binding to this membrane fraction (Fig 4E and Fig S4D). Thus, we concluded that S155 regulated the GEF activity, while T280 regulated the localization of Grp1. We also sought in vivo support for these findings. In one experiment, we isolated the different forms of Grp1 that had been transfected in adipocytes, and then assessed their ability to active ARF6 in the GEF assay. The results confirmed that mutations at S155 affected the GEF activity, while mutations at T280 did not have a similar effect (Fig 4F). In another experiment, we pursued confocal studies and found that mutations at T280 affected the ability of Grp1 to localize to the glut4-positive compartment, while mutations at S155 did not have a similar effect (Fig 4G). Thus, these results further confirmed that one key residue (S155) controlled the GEF activity, while the other key residue (T280) controlled the localization of Grp1 to the glut4-positive endosomal compartment.

We also noted that, although the above effects of Grp1 mutants in the reconstitution system suggested that its phosphorylation was critical for glut4 vesicle formation, we had found above that membrane and cytosol fractions collected from adipocytes in the basal condition

(which should result in low level of phosphorylated Grp1) supported a robust level of glut4 vesicle formation (see Fig 2E). In considering a reconciling explanation, we noted that an ATP-regenerating system was required to reconstitute glut4 vesicle formation (see Fig 2E). Moreover, the in vitro kinase assay indicated that the presence of ATP was sufficient for Akt to phosphorylate Grp1 (see Fig 4C). Taken together, these observations suggested that glut4 vesicle formation occurred in the reconstitution system, because the ATP-regenerating system promoted the ability of Akt to phosphorylate Grp1 in this reconstitution. Confirming this explanation, we isolated Grp1 from the reconstitution system and found that it only became phosphorylated by Akt when the ATP-regenerating system was provided (Fig 4H).

Grp1 activates ARF6 to promote the later steps of glut4 recycling

We also noted that key factors have been identified that actively suppressed the translocation, docking and fusion of glut4 vesicles, which become relieved upon insulin stimulation (Eguez et al., 2005; Jewell et al., 2011; Yamada et al., 2005). As such, how could the activating mutations (S115D and T280D) of Grp1 induce the surface expression of glut4 in the basal condition (see Fig 3B), if Grp1 acted only to promote glut4 vesicle formation? Thus, we next explored whether activation of Grp1 also promoted the later steps of glut4 recycling. Taking a systematic approach, we first noted that the regulation of glut4 recycling could be viewed mechanistically as a hierarchy, with insulin signaling acting at the top, the major transport effectors at the bottom, and key small GTPases in between (Fig S5A). Thus, we first examined whether the Grp1 activating mutants could somehow feedback to activate the insulin signaling cascade. However, assessing the activation status of Akt as a downstream readout for this signaling, we found that the Grp1 mutants did not induce the activation of Akt in the basal (no insulin) condition (Fig 5A). We also confirmed that insulin signaling was intact in cells that expressed the Grp1 mutants, as adding insulin resulted in the activation of Akt (Fig 5A). Thus, we concluded that glut4 recycling induced in the basal condition by the Grp1 mutants did not occur through feedback to activate the insulin signaling cascade.

Next, to examine whether the Grp1 mutants exerted their effect at the level of key small GTPases that governed glut4 recycling, we examined the effect of depleting these small GTPases by siRNA (Fig S5B). We found that only siRNA against ARF6 prevented the Grp1 mutants, either S155D (Fig 5B) or T280D (Fig S5C), from inducing glut4 recycling. We also confirmed that siRNA against RalA and Rab10 were effective, as targeting either small GTPase in control cells inhibited glut4 recycling induced by insulin (Fig S5D). Thus, we concluded that glut4 recycling induced by the activating mutants of Grp1 required ARF6, but not RalA or Rab10.

We then noted that activated ARF6 has been shown previously to interact with the exocyst to promote endocytic recycling in the context of membrane ruffle formation (Prigent et al., 2003). Thus, we pursued the possibility that activation of ARF6 interacted with key effectors for the later steps of glut4 recycling in explaining how the Grp1 mutants could promote all the steps of glut4 recycling. First, we confirmed that glut4 recycling induced by the Grp1 mutants in the basal condition required the exocyst, as siRNA against Sec10 reduced the ability of either S155D (Fig 5C) or T280D (Fig S5E) to induce glut4 recycling. We then sought to detect the activated form of ARF6 in association with key effectors that acted in the later steps of glut4 recycling. For this goal, we again used the GGA domain to probe cell lysates, reasoning that it could potentially detect activated ARF6 in association with another effector, because ARF small GTPases have been suggested to be capable of interacting with more than one effector partner simultaneously (Goldberg, 1999). Indeed, we found that the GGA domain could detect ARF6 in association with either Sec10 or Myo1c (Fig 5D). Importantly, these in vivo interactions required ARF6, as siRNA against ARF6 prevented these interactions (Fig 5E). Thus, these results suggested that Grp1 also promoted the later

steps of glut4 recycling by activating ARF6 to engage core effectors that mediated these steps.

We also noted that two recycling pathways exist in adipocytes, an insulin-responsive one (which transports cargoes such as glut4) and a largely insulin-independent one (which transports “generic” cargoes such as TfR) (Govers et al., 2004; Xiong et al., 2010). We initially confirmed that endogenous Grp1 acts in glut4 recycling, as siRNA against Grp1 did not affect TfR recycling (Fig 6A). We then also sought confirmation that the activating mutants of Grp1 did not promote the surface expression of glut4 through diversion into the “generic” recycling pathway. For this goal, we noted that the Vamp2 SNARE acts in glut4 recycling (Williams and Pessin, 2008), while Vamp3 (also known as cellubrevin) acts in TfR recycling (Daro et al., 1996), which marks the generic pathway. We first confirmed that siRNA against Vamp2 in control adipocytes inhibited insulin-stimulated glut4 recycling (Fig S6A), while siRNA against Vamp3 did not affect this recycling (Fig S6B). Conversely, TfR recycling was inhibited by siRNA against Vamp3, but not against Vamp 2 (Fig S6C). We then found that the ability of the Grp1 mutants to induce glut4 recycling in the basal condition became inhibited, when adipocytes were treated with siRNA against Vamp2 (Fig 6B). In contrast, siRNA against Vamp3 did not have a similar effect (Fig 6C). We also found that TfR recycling was not enhanced by the Grp1 mutants (Fig 6D). Thus, the results altogether led us to conclude that Grp1 activation increased the surface expression of glut4 by promoting the specialized (insulin-regulated) recycling pathway in adipocytes, rather than diverting internal glut4 to the generic (constitutive) recycling pathway.

DISCUSSION

Glut4 recycling has been intensively investigated due to the central role that this process plays in regulating the function of glut4, which is a key molecule in glucose homeostasis. The cumulative results over the years have led to the current view that insulin targets primarily the later steps of glut4 recycling, which are the translocation, docking and fusion steps (Foley et al., 2011; Huang and Czech, 2007; Rowland et al., 2011; Watson and Pessin, 2006). However, we now find that insulin also regulates glut4 vesicle formation, the earliest step of glut4 recycling, by targeting Grp1. Importantly, we also find that this targeting of Grp1 results in all subsequent steps of glut4 recycling being promoted. As such, our findings now suggest the need for a major revision in the current view of how insulin regulates glut4 recycling.

Early studies by electron microscopy could detect the accumulation of internal glut4 vesicles in the basal condition (Slot et al., 1991). Our elucidation of how insulin regulates glut4 vesicle formation through Grp1 now suggests a more precise explanation for this observation. We have found that mutations which abrogate the phosphorylation of Grp1 by Akt can still support a low level of glut4 vesicle formation. Such residual production of vesicle formation in the basal condition is predicted to result in the significant accumulation of internal glut4 vesicles over time, because the later steps of glut4 recycling are known to be actively suppressed in this condition (Chen et al., 2007; Eguez et al., 2005; Jewell et al., 2011; Yamada et al., 2005). Moreover, because we have found that insulin can up-regulate glut4 vesicle formation through Akt phosphorylation of Grp1, the full effect of insulin on glut4 recycling is predicted to involve an even greater level of glut4 vesicles being generated than that seen at the basal condition.

We also note that results from live-imaging studies in recent years that have focused on events near the plasma membrane have further fueled the current view that insulin regulates the later steps of glut4 recycling (Bai et al., 2007; Koumanov et al., 2005; Lizunov et al., 2005; Stenkula et al., 2010). However, it is also notable that other recent studies have found

that insulin regulates events of glut4 recycling at the internal endosomal compartments (Fujita et al., 2010; Xu et al., 2011). Notably though, the dominance of the current view that insulin targets the later steps of glut4 recycling has led these recent studies to conclude that insulin stimulates the translocation of glut4 vesicles to the PM, rather than considering the possibility that glut4 vesicle formation may also be targeted. As such, our current findings suggest a fresh perspective to these recent observations.

Although we have found that the activating mutants of Grp1 in the basal (no insulin) condition can bypass key small GTPases that regulate the later steps of glut4 recycling (RalA and Rab10) to induce the surface expression of glut4, we also note that these small GTPases have been shown previously to be required for glut4 recycling stimulated by insulin (Chen et al., 2007; Sano et al., 2007). Thus, how insulin promotes the later steps of glut4 recycling is predicted to involve a more balanced distribution of stimulation through ARF6, RalA, and Rab10. How this balance may occur is suggested by studies on RalA GAP complex (Chen et al., 2011) and AS160 (Eguez et al., 2005). Upon insulin stimulation, these GAPs have been shown to become inhibited, resulting in increased level of activated RalA and Rab10. As such, a likely explanation is that insulin stimulation leads to a more balanced contribution of key small GTPases (ARF6, RalA, and Rab10) in regulating downstream effectors of glut4 recycling.

Such a balance is also suggested by another mechanistic consideration. The activating mutant forms of Grp1 is predicted to be considerably more potent than the endogenous pool of Grp1 that is activated by insulin signaling due to Akt phosphorylation, because the Grp1 mutants cannot undergo dynamic dephosphorylation, while physiologic phosphorylation through insulin signaling should undergo this dynamic process, due to phosphatase activity often countering kinase activity to prevent uncontrolled signaling. Thus, the reduced ability of insulin stimulation to maintain the active form of Grp1 is predicted to lessen the ability of ARF6 to participate in the later steps of glut4 recycling. As such, RalA and Rab10 that also acts in these later steps of glut4 recycling would become more important in the context of insulin signaling.

It is also notable that the dual situation of some glut4 vesicles being able to form at the basal condition and the ability of SecinH3 to inhibit ARF6 activation acutely has provided an invaluable experimental window to further scrutinize the role of ARF6 in the later steps of glut4 recycling. In the basal condition, about half of the internal glut4 has been suggested to be incorporated into vesicles, often referred as glut4 storage vesicles (Foley et al., 2011; Huang and Czech, 2007; Rowland et al., 2011; Watson and Pessin, 2006). Thus, if ARF6 activated by Grp1 acts only in the vesicle formation step, then SecinH3 should only partially inhibit the ability of insulin to promote the surface expression of glut4. Instead, because we have found that SecinH3 has a marked effect in preventing the ability of insulin to stimulate glut4 recycling, this finding represents further functional support that ARF6 plays an important role in promoting not only the vesicle formation step, but also the later steps of glut4 recycling.

On a broader level, our findings also reveal the complexity by which the different steps of vesicular transport can be regulated to achieve regulated transport. Currently, the later steps (vesicle docking/fusion) are thought to be the main targets of such coordination, as exemplified by key models of regulated transport such as the exocytosis of synaptic vesicles and secretory granules (Blott and Griffiths, 2002; Martens and McMahon, 2008). Indeed, insulin-regulated glut4 recycling that represents one of the most complex examples of regulated transport has also been thought to fit into this mode of regulation. However, we have now uncovered the coordination of a broader spectrum of transport steps, with one small GTPase (ARF6) playing a key role in this coordination (summarized in Fig 7). As

such, an intriguing possibility is that similar degrees of complexity may be uncovered when other types of regulated transport are further scrutinized.

EXPERIMENTAL PROCEDURES

Chemicals, proteins, cells, antibodies, plasmids, as well as sequences used for mutagenesis and siRNA are detailed as supplemental information.

In vivo assays

A quantitative microscopy-based assay that measures glut4 recycling by examining the HA-glut4-GFP expressed in differentiated 3T3-L1 adipocytes was performed as described previously (Li et al., 2007). Briefly, cells were starved for 5 hours for the basal condition and insulin (100 nM) was added for 15 minutes for the stimulated condition. The ratio of surface to total glut4 was quantified by detecting surface glut4 through the HA tag (located in an extracellular domain) and total glut4 through emission of GFP.

The glucose uptake assay was performed as described previously (Li et al., 2007). In conditions that used SecinH3, this compound was added to cells (10 μ M) 30 minutes before adding insulin.

TfR recycling was performed as previously described (Bai et al., 2011).

Subcellular fractionation of adipocytes into compartmental membrane versus vesicular fraction has been described previously (Li et al., 2007). Co-precipitation to detect in vivo interactions has also been described previously (Li et al., 2007).

Colocalization studies using laser confocal microscopy followed by quantitation using imaging software (Image J, NIH) were performed as previously described (Li et al., 2007). The distribution of endosomal TfR was detected by steady-state labeling with Tf added exogenously to cells.

To examine the interaction between activated ARF6 and Sec10 or Myo1C, GST-pulldown assay was performed. Briefly, 3T3-L1 adipocytes stably expressing Grp1 mutants were starved for 5 hours. After cell lysis by 1% Triton X100, the post-nuclear supernatant was incubated with GST-fused GGA3-VHS-GAT immobilized on glutathione-beads to detect activated ARF in a pulldown assay, followed by immunoblotting for ARF6, Sec10 and Myo1C.

In vitro assays

ARF6 GEF assay was performed essentially as previously described (Pacheco-Rodriguez et al., 2002). In brief, recombinant Myc-Grp1 on beads was incubated with purified ARF6 (1 μ g) in the presence of 4 mM MgCl₂, 2 mM EDTA, 0.1% Triton X-100 and 20 μ M GTP γ S at 37° C for 15 minutes. GGA3-VHS-GAT as a GST fusion protein was then used to detect activated ARF in a pulldown assay, followed by immunoblotting for ARF6.

The in vitro kinase assay was performed as previously described (Li et al., 2005). Briefly, activated Akt was isolated from cells and then incubated with recombinant forms of Grp1. Phosphorylation was then detected using the phospho-Akt substrate antibody.

In vitro reconstitution of glut4 vesicles was performed as previously described (Xu and Kandror, 2002). Briefly, membrane and cytosol fractions were isolated from adipocytes, and then incubated in the presence of an ATP-regenerating system (unless otherwise stated) along with recombinant forms of Grp1 (1 μ g/ml). After incubation, centrifugation was

performed to segregate compartmental membrane in the pellet and vesicular membrane in the supernatant, followed by immunoblotting for glut4 in the two fractions.

The recruitment of Grp1 to membrane enriched for internal glut4 was assessed as follows. A membrane fraction enriched for internal glut4 was isolated by first binding biotin-labeled mouse Tf (10 ug/ml) to the surface of 3T3-L1 adipocytes in the basal condition for 60 min at 4° C. Cells were then washed and homogenized followed by centrifugation at 2,000 × g for 5 min at 4° C to obtain the post-nuclear supernatant, which was loaded onto a sucrose gradient (20% - 50%) for equilibrium centrifugation (200,000 × g for 20 hours). Fractions were collected and then analyzed by western blotting for the distribution of Tf (marking plasma membrane) and glut4. Fractions enriched for internal glut4 were then pooled and concentrated by centrifugation (16,000 × g for 20 min at 4° C). To study the recruitment of Grp1 to this membrane fraction, different forms of recombinant myc-tagged Grp1 (1 ug/ml) was incubated with the membrane fraction at 37°C for 15 minutes. Centrifugation (16,000 × g for 20 min at 4° C) was then performed and the resulting pellet and supernatant fractions were immunoblotted for Grp1 to detect the membrane-bound pool (pellet fraction).

Statistical analysis

The Student's t-test was performed, using Graphpad Prism, to determine statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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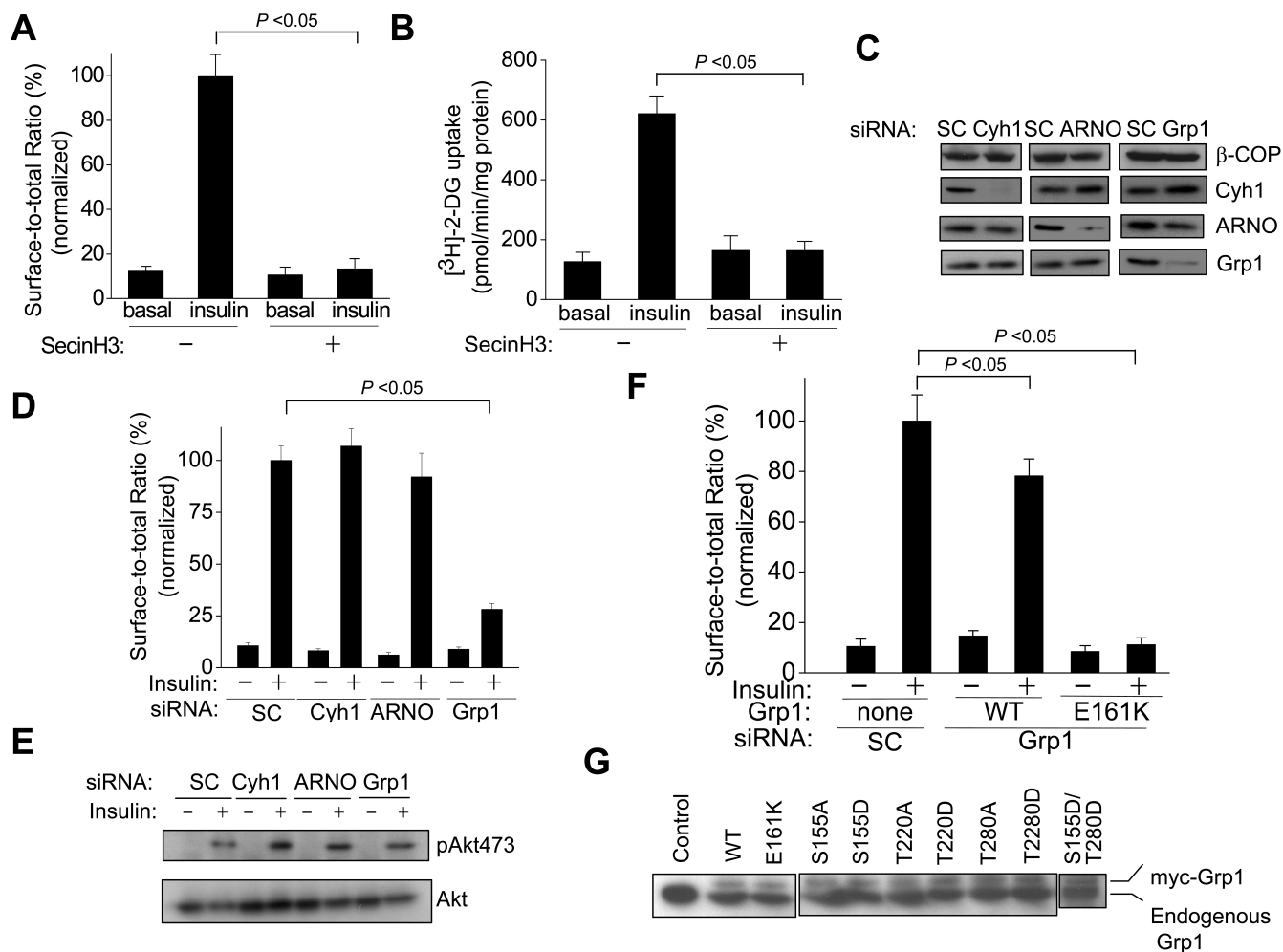
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HIGHLIGHTS

- Grp1 acts as an ARF6 GEF to promote glut4 vesicle formation.
- Grp1 also promotes the later steps of glut4 recycling through ARF6.
- Insulin regulates Grp1 through its phosphorylation by the protein kinase Akt.
- Phosphorylation of Grp1 regulates its GEF activity and membrane recruitment.

**Figure 1.**

Grp1 acts in GLUT4 recycling.

A. The microscopy-based assay was used to quantify GLUT4 recycling, comparing all conditions to insulin stimulation in control cells. The mean with standard error from three experiments is shown.

B. The glucose uptake assay was performed. The mean with standard error from three experiments is shown.

C. Cell lysates were immunoblotted for proteins as indicated, with β -COP level used as loading control.

D. The microscopy-based assay was performed as described above (in A).

E. Cell lysates from different conditions were immunoblotted for proteins as indicated.

F. The microscopy-based assay was performed as described above (in A).

G. Cell lysates were immunoblotted with the anti-Grp1 antibody. See also Figure S1.

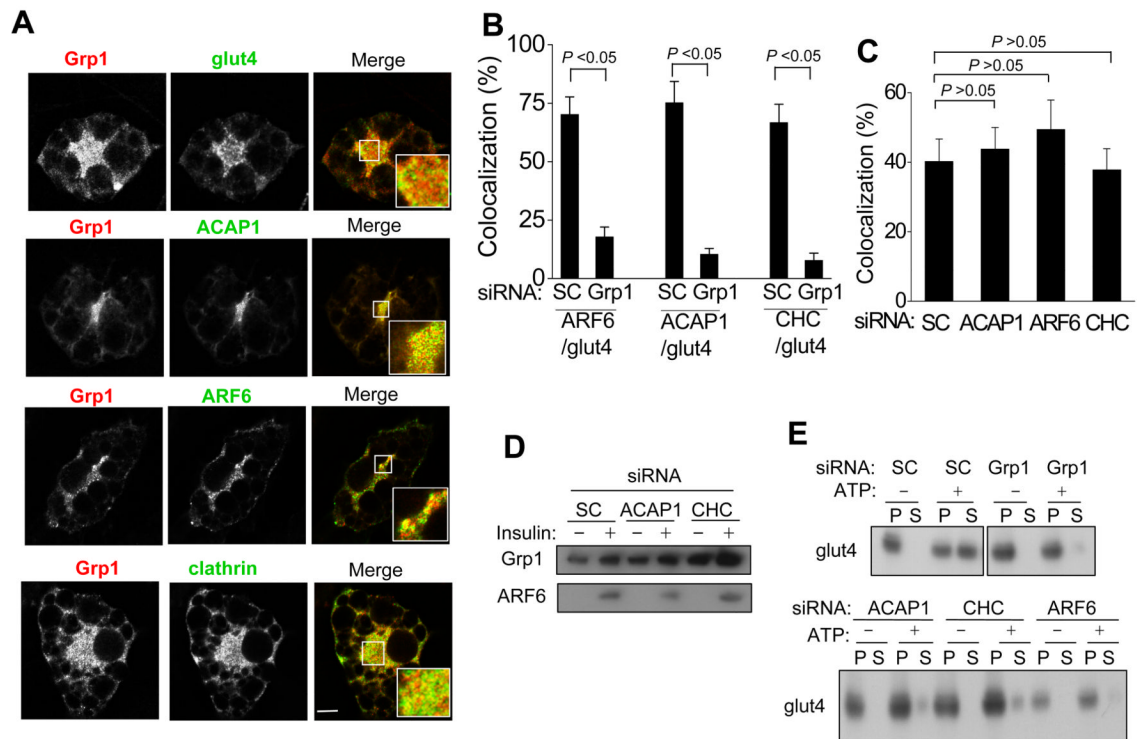


Figure 2.

Grp1 regulates ARF6 and the clathrin-ACAP1 coat in glut4 recycling.

A. Confocal microscopy was performed with Grp1 shown in red and other proteins (as indicated) shown in green; bar, 10 μ m.

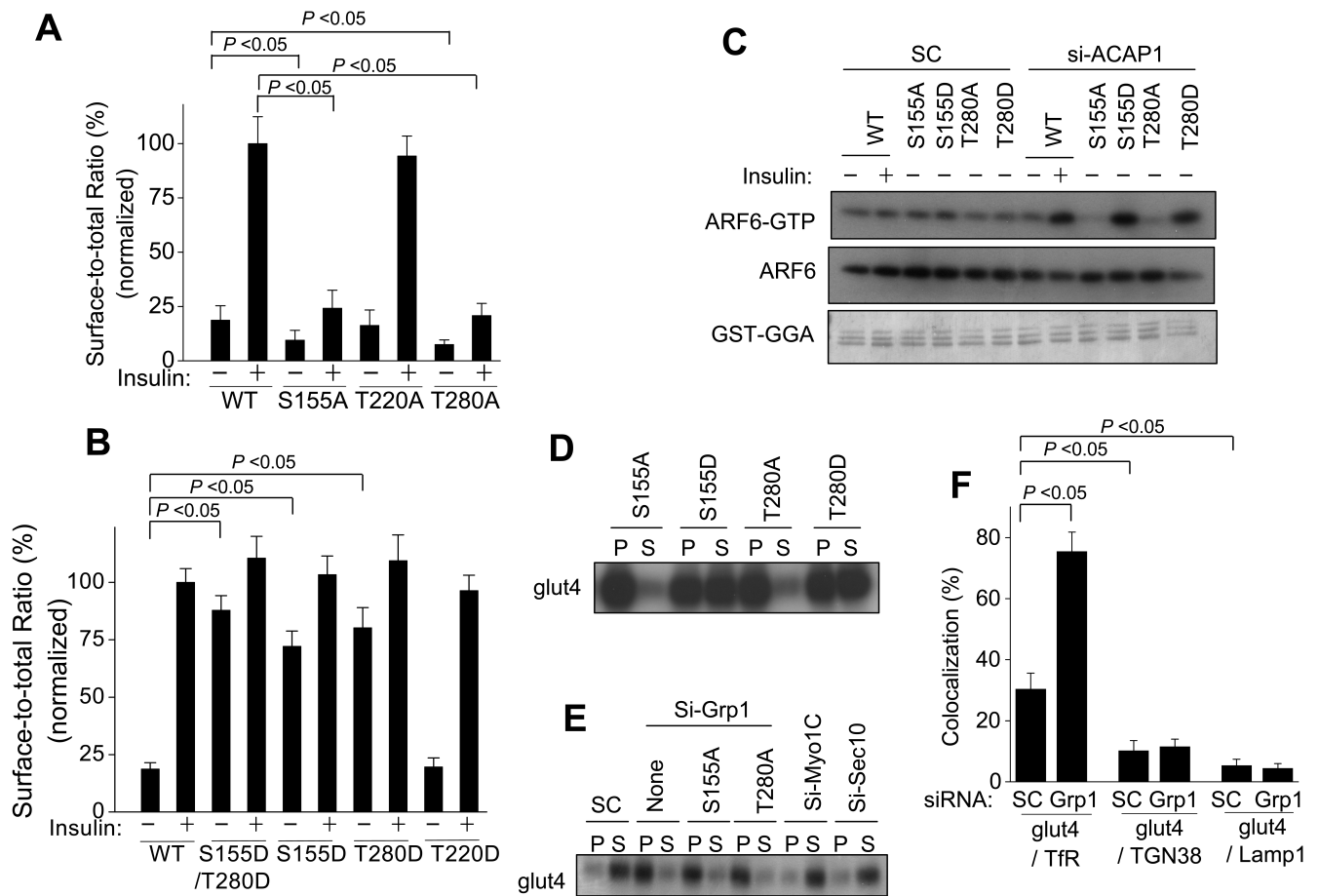
B. Confocal microscopy was performed in the basal condition, followed by quantitation for the fraction of ARF6, ACAP1, or CHC that colocalized with internal glut4. The mean with standard error from three experiments is shown.

C. Quantitative confocal microscopy was performed as described above (in B).

D. The co-precipitation approach was used to detect interaction between Grp1 and ARF6.

E. The glut4 vesicle reconstitution assay was performed, with centrifugation segregating compartmental (in pellet, P) from vesicular (in supernatant, S) membrane fractions.

See also Figure S2.

**Figure 3.**

Insulin regulates Grp1 through Akt phosphorylation.

A. The microscopy-based assay was used to quantify glut4 recycling, comparing all conditions to insulin stimulation in adipocytes expressing the wild-type Grp1. The mean with standard error from three experiments is shown.

B. The quantitative microscopy assay was performed as described above (in A).

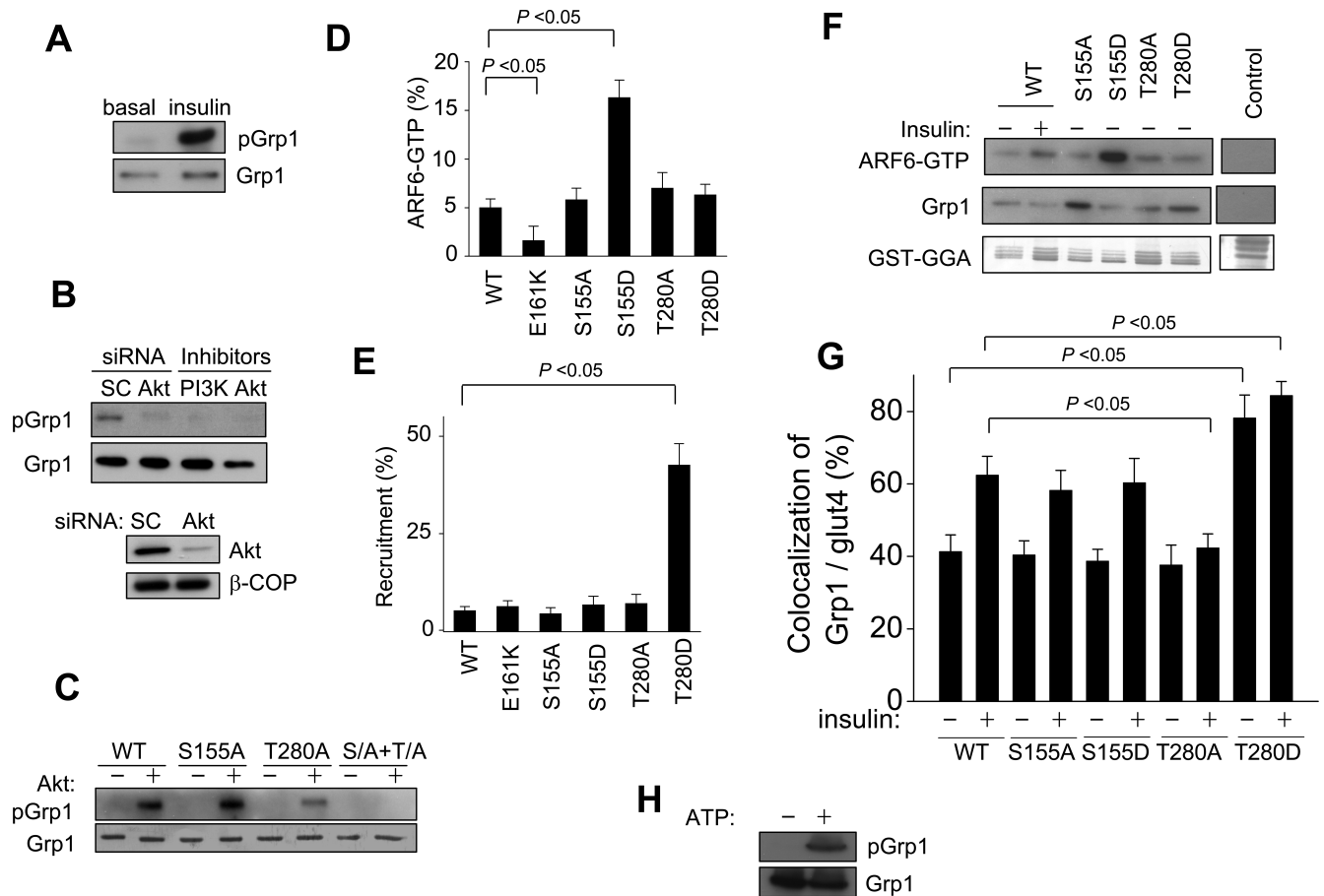
C. Cell lysates were probed with the GGA domain construct and then immunoblotted for proteins as indicated.

D. The glut4 vesicle reconstitution assay was performed by incubating membrane with cytosol that were derived from adipocytes treated with siRNA against Grp1, followed by different forms of Grp1 added as recombinant proteins.

E. Adipocytes were fractionated into vesicular versus compartmental membranes, followed by immunoblotting for glut4.

F. Quantitative confocal microscopy was performed to assess the colocalization of glut4 with different compartmental markers. The mean with standard error from three experiments is shown.

See also Figure S3.

**Figure 4.**

Phosphorylation at distinct residues of Grp1 regulates distinct activities.

A. A myc-tagged form of wild-type Grp1 stably expressed in adipocytes was immunoprecipitated using an anti-myc antibody, and then immunoblotted using the phospho-Akt substrate antibody.

B. Immunoprecipitation followed by immunoblotting was performed as described above (in A), examining cells that had been treated with different conditions as indicated (upper panel). Efficiency of siRNA was documented by immunoblotting of cell lysates (bottom panel).

C. Purified active Akt was incubated with different forms of recombinant Grp1 as indicated for the in vitro kinase assay, followed by immunoblotting using the phospho-Akt substrate antibody.

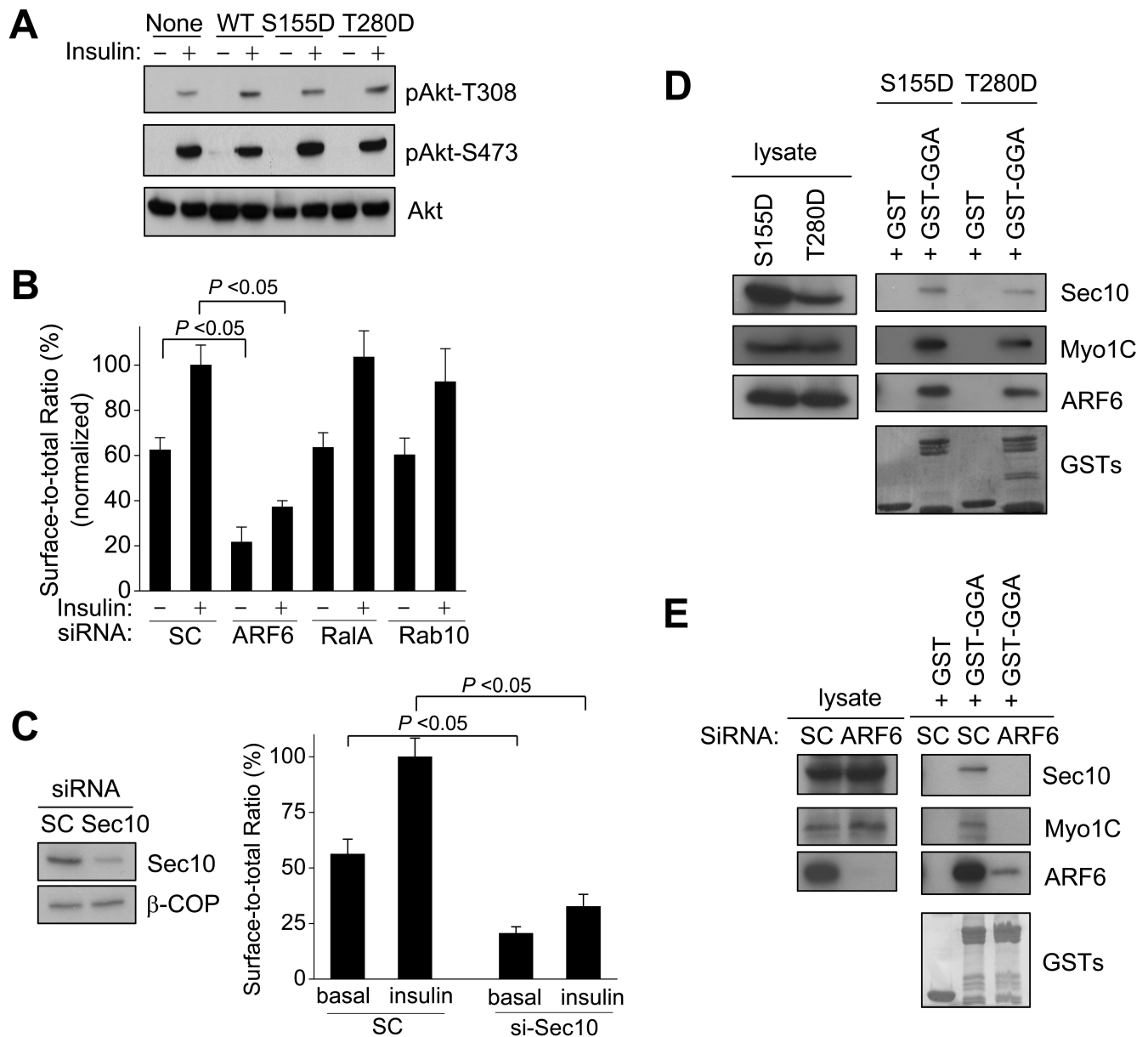
D. The in vitro GEF assay was performed. The mean with standard error from three experiments is shown.

E. The recruitment of different recombinant forms of Grp1 to an endosomal membrane fraction was assessed. The mean with standard error from three experiments is shown.

F. The different forms of Grp1 were isolated from stably expressing adipocytes, and then analyzed by the in vitro GEF assay.

G. Quantitative confocal microscopy was performed to assess the colocalization of different Grp1 mutants with internal glut4. The mean with standard error from three experiments is shown.

H. The glut4 vesicle reconstitution system was performed either in the presence or absence of the ATP-regenerating system. Grp1 was then isolated and then assessed for phosphorylation by Akt using the phospho-Akt substrate antibody. See also Figure S4.

**Figure 5.**

Elucidating how the Grp1 mutants induce glut4 recycling in the basal condition.

A. Cell lysates were immunoblotted for proteins as indicated.

B. The microscopy-based assay was used to quantify glut4 recycling, comparing all conditions to insulin stimulation in control cells. The mean with standard error from three experiments is shown.

C. The quantitative glut4 recycling assay was performed as described above (in A). The mean with standard error from three experiments is shown (right panel). Efficiency of siRNA was also documented (left panel).

D. Cell lysates were incubated with GST-GGA for a pulldown experiment, followed by immunoblotting for ARF6, Myo1c, or Sec10. GST fusion proteins were detected by Coomassie staining.

E. A similar pulldown experiment was performed as described above (in D) for other conditions as indicated.
See also Figure S5.

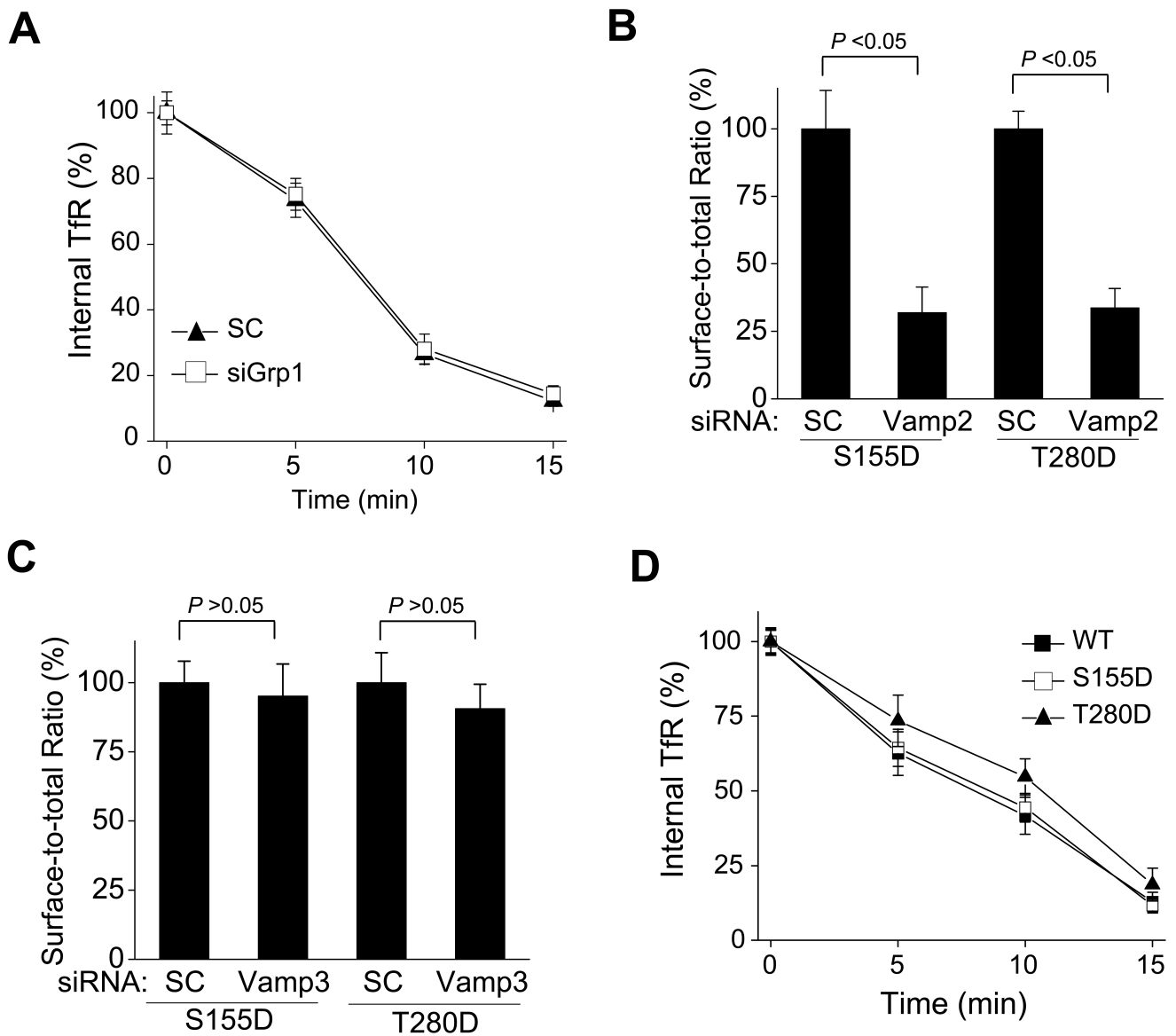


Figure 6.

Grp1 mutants do not induce GLUT4 recycling through the generic recycling pathway.

A. The TfR recycling assay was performed. The mean with standard error from three experiments is shown. No significant difference is observed between the two conditions across all time points ($p > 0.05$).

B. The microscopy-based assay was used to quantify GLUT4 recycling. The mean with standard error from three experiments is shown.

C. The microscopy-based GLUT4 recycling assay was performed as described above (in B).

D. The TfR recycling assay was performed. The mean with standard error from three experiments is shown. No significant difference is observed among the conditions across all time points ($p > 0.05$).

See also Figure S6.

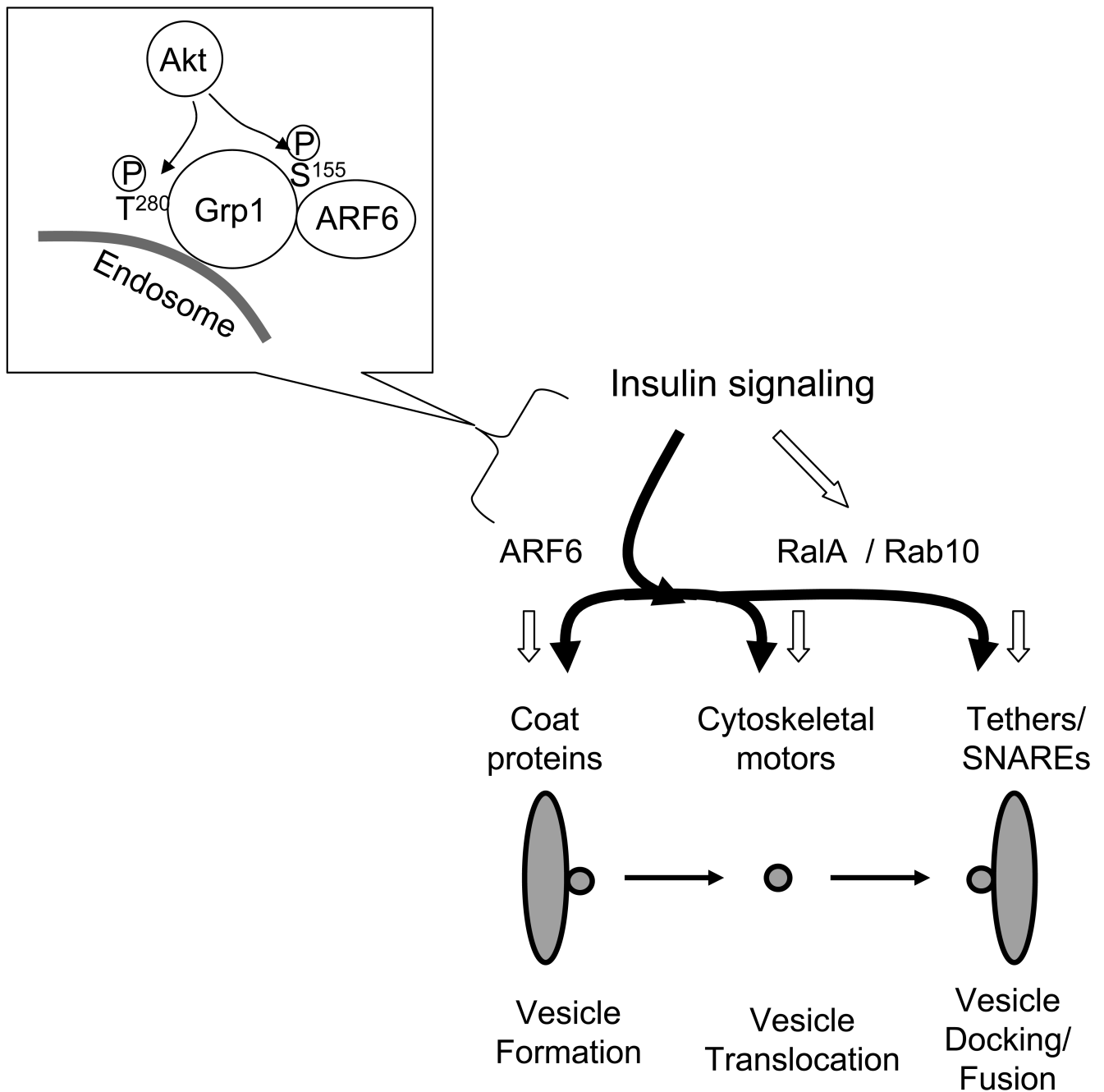


Figure 7. Summarizing how insulin signaling regulates glut4 recycling. The vertical unfilled arrows reflect the current knowledge of how insulin regulates glut4 recycling through key small GTPases. The vertical filled arrows highlight regulatory mechanisms uncovered in this study. Inset shows key residues in Grp1 phosphorylated by Akt, which affects either the catalytic activity or membrane recruitment of Grp1.