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Gapex-5, a Rab31 Guanine Nucleotide Exchange Factor that Regulates Glut4 Trafficking in Adipocytes

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Summary

Insulin stimulates glucose uptake by promoting translocation of the Glut4 glucose transporter from intracellular storage compartments to the plasma membrane. In the absence of insulin, Glut4 is retained intracellularly, although the mechanism underlying this process remains uncertain. Using the TC10-interacting protein CIP4 as bait in a yeast two-hybrid screen, we cloned a RasGAP and VPS9 domain-containing protein, Gapex-5/RME-6. The VPS9 domain is a guanine nucleotide exchange factor for Rab31, a Rab5 subfamily GTPase implicated in *trans*-Golgi Network (TGN)-to-endosome trafficking. Overexpression of Rab31 blocks insulin-stimulated Glut4 translocation, whereas knockdown of Rab31 potentiates insulin-stimulated Glut4 translocation and glucose uptake. Gapex-5 is predominantly cytosolic in untreated cells; its overexpression promotes intracellular retention of Glut4 in adipocytes. Insulin recruits the CIP4/Gapex-5 complex to the plasma membrane, thus reducing Rab31 activity and permitting Glut4 vesicles to translocate to the cell surface, where Glut4 docks and fuses to transport glucose into the cell.

Introduction

Insulin stimulates glucose transport into striated muscle and adipocytes by increasing the concentration of the facilitative glucose transporter Glut4 at the plasma membrane (Bryant et al., 2002; Saltiel and Kahn, 2001). In the absence of insulin, or following inactivation of its receptor, Glut4 undergoes endocytosis (Corvera et al., 1994; Garippa et al., 1996). Approximately half of intracellular Glut4 resides in general endocytotic compartments that contain the transferrin receptor, while the other half is found in a specialized Glut4 storage compartment which lacks endocytotic markers (Martin et al., 1996; Zeigerer et al., 2002), but contains markers of the *trans*-Golgi network (TGN), such as AP-1 and Syntaxins 6 and 16 (Gillingham et al., 1999; Martin et al., 2000; Shewan et al., 2003). Recent studies suggest that the two pools of Glut4 are in equilibrium with each other through a continuous cycle of budding and fusion that promotes intracellular retention of the transporter (Karylowski et al., 2004). Insulin permits escape from this futile cycle, thereby stimulating exocytosis of Glut4 vesicles to the cell surface.

The Rab family of small G-proteins plays an essential role in various cellular trafficking processes (Stenmark and Olkkonen, 2001), including the trafficking of Glut4 vesicles (Dugani and Klip, 2005). Rab proteins cycle between an inactive (GDP-bound) and an active (GTP-

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bound) state, regulated by GTPase activating proteins (GAPs) that promote GTP hydrolysis and guanine nucleotide exchange factors (GEFs) that promote exchange of GDP for GTP. The critical role of Rab proteins in vesicle trafficking suggests that they may represent a site of integration for signals from the insulin receptor.

The stimulation of glucose transport by insulin in adipocytes is thought to require two distinct signaling pathways. Upon activation, the insulin receptor catalyzes the tyrosine phosphorylation of the IRS family of adaptor proteins, which results in recruitment and activation of phosphatidylinositol 3-kinase (PI3K), and downstream activation of PIP_3-dependent processes (Kelly and Ruderman, 1993; Kelly et al., 1992). Although this pathway is required for Glut4 translocation, substantial evidence suggests that it is not sufficient (reviewed in Saltiel and Pessin, 2002). The insulin receptor also catalyzes the tyrosine phosphorylation of the adapter protein APS (Liu et al., 2002), resulting in the recruitment and tyrosine phosphorylation of c-Cbl or Cbl-b (Liu et al., 2003), and the subsequent activation of the Rho family GTPase TC10 (Chiang et al., 2001). The absolute requirement for this pathway is uncertain. Expression of dominant negative mutants of most of these proteins block glucose transport (Baumann et al., 2000; Chiang et al., 2001; Liu et al., 2003; Liu et al., 2002), while knockdown of CAP, Cbl and APS have led to inconsistent effects (Ahn et al., 2004; Mitra et al., 2004). In this regard, we have recently shown that upon lowering Glut1 levels, knock down of CAP (Chiang et al, in preparation) or TC10 (Chang et al., 2006) blocks insulin-stimulated glucose uptake and Glut4 translocation.

Once activated, TC10 recruits a number of effectors that participate in insulin-stimulated Glut4 trafficking, docking and fusion, including the exocyst protein Exo70 (Inoue et al., 2003) and the multifunctional adaptor protein CIP4 (Cdc42 interacting protein 4) (Chang et al., 2002). CIP4 interacts with the active, GTP-loaded forms of Cdc42 (Aspenstrom, 1997) and TC10 (Chang et al., 2002). The N-terminal FCH domain of CIP4 resembles the non-kinase domain of the FER and Fes/Fps family of tyrosine kinases and contains a BAR domain (Itoh et al., 2005). CIP4 also harbors two central coiled-coil motifs and a C-terminal SH3 domain (Aspenstrom, 1997). We previously reported that overexpressed CIP4 is predominantly found in an intracellular compartment in adipocytes under basal conditions, and is recruited to the plasma membrane following insulin stimulation (Chang et al., 2002). TC10 activation is both necessary and sufficient for this translocation of CIP4. The FCH domain, which has been shown to interact with microtubules (Tian et al., 2000), is necessary for translocation to the plasma membrane (Chang et al., 2002), suggesting that movement of CIP4 might proceed along a microtubule tract. Overexpression of mutant forms of CIP4 that cannot translocate to the plasma membrane or interact with TC10 block insulin-stimulated Glut4 translocation. In contrast, a mutant of CIP4 lacking the SH3 domain is constitutively localized to the plasma membrane and has no effect on Glut4 translocation (Chang et al., 2002). These data suggest that CIP4 is an adaptor protein that recruits additional molecules to the plasma membrane, some of which may participate in the regulation of Glut4 trafficking in response to insulin.

To better understand the role of CIP4 in insulin-stimulated Glut4 translocation, we screened for CIP4-interacting proteins using the yeast-two hybrid assay system. Here we describe one such binding partner, Gapex-5, an evolutionarily conserved multi-domain protein that activates Rab31, a Rab5 subfamily GTPase that influences the trafficking of Glut4 vesicles in adipocytes.

Results

Identification and expression of mouse Gapex-5

To better understand its role in the regulation of insulin-stimulated glucose uptake, we used CIP4 as bait to screen a yeast two-hybrid cDNA library derived from 3T3-L1 adipocytes. A 1.6 kb fragment was identified encoding a novel protein lacking the N- and C-termini. The

full-length sequence was assembled by using the sequence of the yeast two-hybrid fragment to search for overlapping ESTs in the NCBI database. A 4.3 kb full-length cDNA was cloned by RT-PCR using total RNA from 3T3-L1 adipocytes. The reading frame started with an ATG codon in a Kozak consensus sequence and encoded a protein of 1437 amino acids (Figure S1). Sequence analysis using the NCBI database revealed that protein encoded by this cDNA is evolutionarily conserved, and contains an N-terminal Ras GTPase activating protein (GAP) domain, a C-terminal VPS9 domain (containing a Rab5 guanyl nucleotide exchange factor), and a central PXXP sequence similar to other SH3 domain-binding sequences (Figure 1A and Figure S1). We thus named the protein Gapex-5. The *C. elegans* homologue of Gapex-5 called RME-6 and the human homologue called RAP6 were recently described as Rab5 exchange factors (Hunker et al., 2006; Sato et al., 2005). The human and rat orthologues were 90% identical to the mouse gene, and the RasGAP, proline-rich and VPS9 domains showed 97%, 100%, and 100% identity respectively. Orthologues identified in *D. melanogaster*, *C. elegans* and *G. gallus* were 33%, 30%, and 83% identical to the mouse sequence, respectively. However, the genes found in these organisms did not contain the PXXP sequence. Therefore, this motif may play an important role in regulating the mammalian isoforms.

To examine the tissue distribution of Gapex-5, we performed multi-tissue Northern blot analysis using mouse total RNA from various tissues with the 1.6 kb yeast-two hybrid prey as the probe. As shown in Figure 1B, the Gapex-5 transcript is ubiquitously expressed, although the level of expression varied in different tissues, and was most highly expressed in heart, liver, kidney and testes. We also compared the expression of Gapex-5 in 3T3-L1 fibroblasts (pre-adipocytes) and fully differentiated adipocytes by Northern (Figure 1C) and western blot (Figure 1D) using an anti-Gapex-5 polyclonal antibody. Both Northern and western blot analyses revealed that Gapex-5 expression increases modestly upon differentiation of fibroblasts to adipocytes.

Interaction of Gapex-5 with CIP4

A series of truncation mutants were generated to evaluate the interaction of Gapex-5 with CIP4 (Figure 2A). The Gapex-5 fragment isolated in the yeast two-hybrid screen (residues 437–972) was expressed as a GST-fusion protein and used to pull-down CIP4 and its mutants ectopically expressed in Cos-1 cells. GST-Gapex-5 interacted with full-length CIP4 and a CIP4 mutant lacking the N-terminal FCH domain, but did not interact with the mutant of CIP4 lacking the SH3 domain (Figure 2B). These data suggest that the central proline-rich region in the yeast two-hybrid fragment of Gapex-5 is required for interaction with CIP4.

The interaction between Gapex-5 and CIP4 was further examined by co-immunoprecipitation. Myc-tagged wild-type CIP4 or various mutants were overexpressed either alone or in combination with HA-Gapex-5 in Cos-1 cells as indicated in Figure 2C. Lysates were immunoprecipitated with an anti-HA antibody, followed by immunoblotting with an anti-myc antibody, revealing that CIP4 co-immunoprecipitates with Gapex-5. Consistent with the GST pull-down results, CIP4ΔSH3 did not co-immunoprecipitate with Gapex-5. Conversely, a Gapex-5 mutant lacking the proline-rich motif (ΔPXXP) failed to interact with CIP4. These data, combined with the GST pull-down assay, indicate that the SH3 domain of CIP4 specifically interacts with the PXXP motif of Gapex-5.

CIP4 recruits Gapex-5 to the plasma membrane in response to insulin

We reported previously that exogenously expressed CIP4 translocates to the plasma membrane of adipocytes in response to insulin stimulation (Chang et al., 2002). We sought to confirm these results with endogenous CIP4 in adipocytes by immunofluorescence using a monoclonal antibody against CIP4. In the absence of insulin, endogenous CIP4 displayed punctate intracellular staining, as well as some plasma membrane localization. Insulin stimulated the

accumulation of endogenous CIP4 at the cell surface in a manner similar to the expressed protein (Figure 2D).

To determine whether CIP4 affects the localization of Gapex-5, we overexpressed myctagged CIP4 and HA-tagged Gapex-5 in adipocytes and examined their localization by confocal microscopy and quantified the amount of Gapex-5 present at the plasma membrane (Figure 2E and 2F). When overexpressed alone, Gapex-5 exhibited a predominantly cytoplasmic localization that was unchanged by exposure of cells to insulin. However, when co-expressed with CIP4, both proteins localized to intracellular compartments in the absence of insulin, and translocated to the plasma membrane in response to insulin stimulation. When co-expressed with CIP4ΔN, the mutant of CIP4 lacking the FCH/BAR domain required for translocation to the plasma membrane (Chang et al., 2002), Gapex-5 failed to translocate to the plasma membrane in response to insulin, although the basal level of Gapex-5 at the plasma membrane was slightly elevated. Gapex-5 also failed to translocate in response to insulin when co-expressed with CIP4ΔSH3, a mutant of CIP4 that is constitutively localized at the plasma membrane and lacks the Gapex-5 interaction domain. Together, these data suggest that Gapex-5 constitutively interacts with CIP4, and further that CIP4 recruits Gapex-5 to the plasma membrane in response to insulin, presumably via TC10 activation. Furthermore, the finding that ectopically expressed Gapex-5 only translocates to the cell surface when CIP4 is also overexpressed suggests that CIP4 is absolutely required for the regulation of Gapex-5 localization by insulin. When overexpressed alone, Gapex-5 exists in vast excess to endogenous CIP4, thereby preventing the visualization of insulin-stimulated Gapex-5 translocation.

To determine whether TC10 activation is required for the recruitment of the CIP4/Gapex-5 complex to the plasma membrane, we transfected adipocytes with myc-Gapex-5 and eGFP-CIP4, together with HA-tagged wild-type or constitutively active ($Q^{75}L$) TC10 or empty vector. After 24 hr, the cells were immunostained and analyzed by confocal microscopy. The CIP4/Gapex-5 complex only translocated to the plasma when TC10 ($Q^{75}L$) was co-expressed, indicating that TC10 activation is sufficient for CIP4/Gapex-5 translocation (Figure S2).

The VPS9 domain of Gapex-5 interacts with GDP-bound Rab5 and Rab31

The VPS9 domain, together with an upstream tandem helical bundle (HB) region, is a specific GDP/GTP exchange module for the Rab5 subfamily of G proteins (Carney et al., 2006; Delprato et al., 2004), which includes Rab5, Rab21, Rab22a and Rab31 (also known as Rab22b) (Stenmark and Olkkonen, 2001). The HB-VSP9 tandem of Gapex-5 is homologous to the corresponding regions of known activators of Rab5 (Figure 3A). To determine the Rab binding specificity of Gapex-5, we incubated lysates of Cos-1 cells overexpressing Rab5a, Rab22a or Rab31 with GDP or GTPγS and performed pull-down assays using a GST-tagged HB-VPS9 domain of Gapex-5. Although the VPS9 domain interacted with GDP-bound Rab5 and Rab31, the affinity was markedly stronger for Rab31. Interestingly, the VPS9 domain did not interact with Rab22a, thus differentiating Rab31 from Rab22a (Figure 3B).

Gapex-5 is a GEF for Rab31

Although Rab31 is a Rab5 family member, there is little information about downstream effectors, and we thus sought to identify Rab31 binding proteins. We incubated a GST-Rab31 fusion protein with GDP or GTPγS, followed by addition of lysates from Cos-1 cells. Precipitated proteins were separated by SDS-PAGE and stained with Coomassie blue. A 180 kDa protein was identified that preferentially interacted with GTPγS-bound Rab31 (data not shown). To determine if this protein corresponds to the Rab5 effector EEA1 (early endosome antigen 1) (Simonsen et al., 1998), we immunoblotted the precipitated proteins with antibodies against EEA1, and found that endogenous EEA1 is the 180kDa Rab31-binding protein (Figure

3C). Surprisingly, EEA1 had greater affinity for Rab31 than for Rab5. EEA1 has two Rab binding domains, one close to the amino terminus and another at the carboxyl terminus (Simonsen et al., 1998). GTP-bound Rab31 interacted strongly with both domains, whereas Rab5 displayed stronger affinity for the amino terminal domain (Figure 3D), as previously reported (Simonsen et al., 1998). We also examined the interaction of Rab31 with the Rab5 effector Rabaptin-5 (Stenmark et al., 1995). Rabaptin-5 failed to bind to Rab31, although the protein did interact with Rab5 (Figure 3C).

To determine whether Gapex-5 is required for GTP loading on Rab31 *in vivo*, we knocked down Gapex-5 in HEK-293 cells, and assayed Rab31 activity by pull-down with EEA1. Knockdown of Gapex-5 with siRNA oligo reduced the activation state of Rab31 by almost 60% (Figure 3E and 3F), suggesting that Gapex-5 is a GEF for Rab31, and required to maintain its activation state in these cells.

Gapex-5 overexpression blocks the insulin-stimulated Glut4 translocation

Because Gapex-5 is constitutively associated with CIP4, a protein previously implicated in regulating insulin-stimulated Glut4 translocation (Chang et al., 2002), we overexpressed full-length Gapex-5 and its deletion mutants to examine their role in the regulation of Glut4 translocation (Figure 4). 3T3-L1 adipocytes were transfected with Glut4-eGFP, along with HA-tagged full-length Gapex-5, its ΔGAP or ΔHB–VPS9 mutants or an empty vector. All of these constructs could be detected in adipocytes by immunoblot analysis (Figure 4A). Cells were treated with or without insulin, and the translocation of Glut4 was evaluated by cell surface fluorescence (Figure 4B). In the basal state, fewer than 5% of the cells exhibited plasma membrane eGFP fluorescence, whereas in response to insulin approximately 70% of the cells showed plasma membrane fluorescence. Interestingly, when Glut4-eGFP was co-expressed with Gapex-5, only 30% of the cells underwent insulin-stimulated translocation of Glut4. Overexpression of the Gapex-5 mutant lacking the GAP domain also blocked Glut4 translocation; however, the ΔHB-VPS9 mutant was only minimally inhibitory, far less than what was observed for the full-length or the ΔGAP protein (Figure 4C).

Overexpression of Gapex-5 could potentially inhibit the plasma membrane localization of Glut4 by blocking the insulin-stimulated exocytosis of Glut4, or accelerating its internalization from the cell surface. To distinguish between these possibilities, we used a Glut4 mutant that cannot efficiently undergo endocytosis from the plasma membrane. Mutations in the N-terminal F^5QQI^8 motif of Glut4, particularly substitution of phenylalanine to alanine (F^5A), results in accumulation of Glut4 at the plasma membrane due to a decreased rate of endocytosis (Garippa et al., 1994; Piper et al., 1993). Interestingly, Gapex-5 overexpression inhibited both basal and insulin-stimulated plasma membrane localization of the F^5A mutant (Figure 4D). Thus, it is possible that the primary function of Gapex-5 with respect to Glut4 trafficking is to regulate translocation of the Glut4-containing vesicle from intracellular compartments to the cell surface. However, we acknowledge that these experiments do not directly demonstrate an effect on exocytosis.

Although the precise mechanism by which Glut4 is internalized from the cell surface is not clear, it is thought that Glut4 undergoes endocytosis through clathrin-coated pits in a manner similar to the transferrin receptor (Robinson et al., 1992; Slot et al., 1991). Overexpression of Gapex-5 did not affect the uptake of Texas Red-conjugated transferrin in 3T3-L1 adipocytes (data not shown). To determine directly whether Gapex-5 can regulate endocytosis of Glut4 from the cell surface, we transfected adipocytes with a Glut4 construct possessing an exofacial myc epitope tag inserted between transmembrane domains 1 and 2 (Kanzaki et al., 2001), and determined the percentage of cells with plasma membrane and/or intracellular staining of myc. Interestingly, Gapex-5 overexpression did not increase the number of cells with intracellular myc label (Figure S3). These data, combined with the effect of Gapex-5 on endocytosis-

defective Glut4, indicate that Gapex-5 probably does not substantially influence at least the initial endocytosis of Glut4. However, it is conceivable that Gapex-5 could play a role in subsequent endocytotic steps, perhaps including fusion of clathrin-coated vesicles with early endosomes.

Plasma membrane targeting of Gapex-5 relieves the inhibition of Glut4 translocation

Overexpressed Gapex-5 is predominantly intracellular in the basal state and is recruited to the cell surface by CIP4 in response to insulin stimulation (Figure 2). To examine the role of Gapex-5 recruitment to the cell surface in insulin-stimulated Glut4 translocation, we targeted Gapex-5 to the plasma membrane with two different methods, co-expression with CIP4, which permits insulin-stimulated translocation to the plasma membrane (Figure 2), or by introduction of a membrane targeting sequence into Gapex-5. We first performed triple transfections with Glut4-eGFP, Gapex-5 and CIP4. Whereas expression of wild-type Gapex-5 alone blocked Glut4 translocation, co-expression of CIP4 prevented the inhibitory effect of Gapex-5 and rescued the insulin stimulation of Glut4 translocation (Figure 5A and 5B).

To target Gapex-5 to the plasma membrane by an independent method, we also generated a chimera of Gapex-5 linked to the C-terminal region of H-Ras (Figure 5C). H-Ras contains C-terminal sequences that direct tandem palmitoylation and farnesylation of the protein, targeting it to lipid raft microdomains of the plasma membrane (Prior et al., 2001). We previously demonstrated that the last 22 residues of Ras are sufficient for membrane targeting (Watson et al., 2001), leading us to generate a chimera of Gapex-5 linked to these C-terminal residues of Ras. This mutant of Gapex-5, referred to as Gapex-5/CAAX, primarily localizes to the plasma membrane in adipocytes. Overexpression of Gapex-5/CAAX produced only a modest inhibition of insulin-stimulated Glut4 translocation, far less than that observed with WT Gapex-5 (Figure 5D and 5E). These results, combined with the CIP4 co-expression study, indicate that the intracellular localization of Gapex-5 is critical for its ability to block Glut4 translocation, and further that the plasma membrane localization of Gapex-5 induced by insulin relieves the inhibitory effect of Gapex-5 on Glut4 translocation.

Overexpression of Rab31 blocks Glut4 translocation in adipocytes

Although previous studies have shown that Rab31 colocalizes with markers of *trans*-Golgi, TGN and endosomes (Field et al., 2000; Rodriguez-Gabin et al., 2001), its localization in adipocytes has not been described. We thus sought to examine the immunolocalization of HA-Rab31 in adipocytes. Interestingly, the overexpressed Rab31 protein was concentrated in a perinuclear compartment, where it partially colocalized with endogenous EEA1 and syntaxin-6 (Figure S4), markers for early endosomes and TGN, respectively. Because Gapex-5 is a regulator of Rab31, we reasoned that it might maintain intracellular Rab31 in an active state, and thus tested the effect of overexpression of Rab31 and its mutants on Glut4 translocation (Figure 6A). Adipocytes were transfected with wild-type, dominant negative ($S^{19}N$) or constitutively active ($Q^{64}L$) mutants of Rab31 along with Glut4-eGFP, and cells were treated with or without insulin. Dominant-negative Rab31 had no effect on insulin-stimulated Glut4 translocation, but increased basal accumulation of Glut4 at the plasma membrane by 3-fold. In contrast, overexpression of wild-type or constitutively active Rab31 blocked Glut4 translocation (Figure 6A and 6B). To determine if this effect of Rab31 was specific for Glut4, we overexpressed constitutively active Rab31 and examined Glut1 translocation. Interestingly, overexpression of the Rab protein did not block the basal or insulin-stimulated translocation of Glut1 (Figure S5).

Rab31 knockdown potentiates insulin-stimulated Glut4 translocation and glucose uptake

To determine the effect of endogenous Rab31 on Glut4 translocation and glucose uptake, we preformed siRNA-mediated knockdowns in 3T3-L1 adipocytes. The depletion of Rab31 had

no effect on the expression levels of Glut4 and caveolin, nor did it block insulin-stimulated phosphorylation of Akt or Cbl (Figure 6C). Interestingly, the knockdown of Rab31 significantly potentiated the effect of low dose (0.1–10 nM) insulin on glucose transport in adipocytes (Figure 6D). To ensure that this is not an off-target effect, we used a different siRNA oligonucleotide for Rab31 and its own matching scrambled control. Interestingly, knockdown of Rab31 with this siRNA also potentiated insulin-stimulated glucose uptake (Figure S6).

To confirm that the enhanced glucose transport in the face of Rab31 knockdown is due to increased Glut4 translocation, we used adipocytes stably infected with retrovirus expressing exofacial myc-tagged Glut4-eGFP (Bogan et al., 2001). Knockdown of Rab31 in these cells significantly increased Glut4 translocation stimulated by 1 nM insulin. However, as determined by anti-myc staining in unpermeabilized cells, Glut4 did not efficiently fuse with the plasma membrane in every cell at this low dose of insulin (Figure 6E). Quantification of the number of cells with exofacial myc staining revealed that only 12% of control cells had myc epitope exposure in response to 1 nM insulin. In contrast, the knockdown of Rab31 resulted in 22% of cells with exofacial myc staining (Figure 6F), suggesting that Rab31 depletion potentiates the effect of low dose insulin on Glut4 translocation. Higher concentrations of insulin resulted in a more efficient extracellular exposure of Glut4 in both control and knockdown cells (data not shown).

Insulin inhibits the activity of Rab31 in adipocytes

We next investigated whether insulin regulates the activity of Rab31 in adipocytes. Adipocytes were treated with insulin for 0, 5, 10 or 20 min and the activation state of Rab31 was determined by pull down with GST-EEA1. Insulin reduced the GTP-dependent interaction of Rab31 with EEA1 in a time-dependent manner, suggesting that insulin inactivates Rab31 (Figure 7A and 7B). Interestingly, pretreatment of cells with the PI3K inhibitor, wortmannin did not block the inhibitory effect of insulin on Rab31 GTP levels (data not shown).

Discussion

Our previous attempts to understand the contribution of TC10 to the regulation of glucose transport led us to the protein CIP4, a multi-domain adapter that is recruited to the plasma membrane in response to insulin. While overexpression of wild-type CIP4 or a mutant that is constitutively localized to the plasma membrane had no effect, overexpression of CIP4 mutants that fail to translocate to the plasma membrane blocked insulin-stimulated recruitment of Glut4 to the cell surface in adipocytes (Chang et al., 2002). These data suggested that CIP4 may act as an adaptor that recruits additional molecules to the plasma membrane to regulate Glut4 trafficking. Gapex-5 appears to be one such protein. This molecule interacts constitutively with CIP4, is predominantly intracellular, and is recruited to the plasma membrane with CIP4 in response to insulin. Whereas overexpression of Gapex-5 suppresses cell surface localization of Glut4, plasma membrane targeting of Gapex-5 by co-expression of CIP4 or introduction of a plasma membrane targeting sequence relieves the inhibitory effect of Gapex-5 on Glut4 translocation. Together, these data suggest that the intracellular localization of Gapex-5 defines its inhibitory effect on Glut4 trafficking, which is subsequently alleviated by its plasma membrane recruitment in response to insulin.

Gapex-5 is present in organisms as evolutionarily divergent as worms and humans, suggesting that it has a conserved role in protein trafficking. In addition, worms deficient for *rme-6*, the *C. elegans* homolog of Gapex-5, have diminished Rab5 activation and a defect in endocytosis of the yolk receptor, YP170, from the plasma membrane (Sato et al., 2005). Interestingly, unlike the mammalian homologs, Gapex-5 from lower organisms lacks the proline-rich region required for interaction with CIP4. This suggests that in these species, the subcellular

localization of Gapex-5 may not be under the same regulatory control, and that the ability to interact with CIP4 was acquired later in evolution.

VPS9 domain-containing proteins catalyze the GDP/GTP exchange reaction on Rab5 family members (Carney et al., 2006; Delprato et al., 2004), which includes Rab5, Rab21, Rab22a and Rab31 (Stenmark and Olkkonen, 2001). Several GEFs for Rab5 subfamily members have been identified, including Rabex-5 (Horiuchi et al., 1997), Rin1 (Tall et al., 2001) and Alsin (Topp et al., 2004). Gapex-5 appears to be an efficient and specific activator of Rab31. This small GTP-binding protein is 45% identical to Rab5 and 71% identical to Rab22a. Like Rab5 and Rab22a (Kauppi et al., 2002; Simonsen et al., 1998), Rab31 interacts in a nucleotide-dependent manner with EEA1, a multi-domain tethering factor involved in the fusion of endosomes (Simonsen et al., 1998). However, unlike Rab22a, which interact with only the N-terminal Rab binding domain of EEA1 (Kauppi et al., 2002), Rab31 interacts with both the N- and the C-terminal Rab binding domains of EEA1, resulting in higher affinity binding. While Rab31 can associate with EEA1-containing endosomes (Figure S4), overexpression of constitutively active Rab31 does not result in the formation of large hollow endocytotic structures typically observed with the overexpression of constitutively active Rab5 (Stenmark et al., 1994). This suggests that while Rab5 can regulate homotypic endosome fusion (Stenmark et al., 1994), Rab31 probably primarily regulates heterotypic endosome fusion.

Although Rab31 is poorly characterized, it is thought to play a role in TGN-to-endosome trafficking. For example, Rab31 colocalizes with markers of *trans*-Golgi, TGN and endosomes (Field et al., 2000; Rodriguez-Gabin et al., 2001). Furthermore, analysis of live cells using time lapse video microscopy showed that trafficking of vesicles from the TGN to endosomes occurs along microtubule tracks via small tubulo-vesicular organelles that contain Rab31 (Rodriguez-Gabin et al., 2001). The Rab31 effector EEA1 may play an essential role in the fusion of TGN-derived vesicles with endosomes. Whereas EEA1 is primarily associated with homotypic fusion of early endosomes (Mills et al., 1998), a pool of EEA1 may also be involved in fusion of post-Golgi vesicles with endosomes, through a direct interaction of EEA1 with syntaxin-6 (Simonsen et al., 1999), a t-SNARE in the TGN for vesicles arriving from the endosomal system (Mallard et al., 1998).

Intracellular Glut4 is stored in a general endosomal compartment, as well as in a specialized Glut4 storage compartment (GSC), which may be derived from the TGN (Martin et al., 1996; Shewan et al., 2003 ; Watson et al., 2004; Zeigerer et al., 2002). Recent studies suggest that under basal conditions, Glut4 undergoes a futile intracellular cycle between these two compartments (Bryant et al., 2002). By preventing its trafficking to the plasma membrane, this intracellular cycling of Glut4 may be a critical element in the basal retention of Glut4. Indeed, in the absence of insulin, the Glut4 vesicles are excluded from the cell surface by a continuous cycle of budding and fusion with endosomes (Karylowski et al., 2004).

This type of intracellular cycling is not unique to Glut4. For example, the yeast protein chitin synthase III (Chs3p) is also retained intracellularly by continuously cycling between the TGN and early endosomes (Valdivia et al., 2002). Furthermore, the aquaporin-2 water channel may also be retained by undergoing an intracellular cycle in a manner similar to Glut4 (Brown, 2003). Thus, it is possible that for proteins that undergo regulated transport to the plasma membrane, a continuous cycle of budding and fusion of vesicles with endosomes may be a general mechanism to promote intracellular retention of these proteins under conditions in which they are not needed at the plasma membrane. However, it should be noted that for Glut4, additional mechanisms involving a physical tether have also been proposed (Bogan et al., 2003).

TC10 is an insulin-regulated GTPase that recruits multiple effectors that influence various aspects of the Glut4 vesicle trafficking. Besides regulating tethering of Glut4 vesicles at the plasma membrane by recruiting the exocyst complex (Inoue et al., 2003; Inoue et al., 2006), TC10 may also regulate intracellular retention of Glut4 vesicles by recruiting CIP4 to the plasma membrane. Our data suggest that the intracellular retention of Glut4 is controlled by Rab31 and its regulator, Gapex-5. Consistent with the effect of overexpressing Gapex-5, overexpression of the active form of Rab31 blocks translocation of Glut4. However, while the dominant negative Rab31 increases basal Glut4 translocation, overexpression of a Gapex-5 mutant lacking the Rab31 GEF domain (Gapex-5ΔHB–VPS9) does not affect basal Glut4 trafficking. This probably reflects the fact that this mutant of Gapex-5 does not act as a dominant negative inhibitor of Rab31. It would be desirable to have a point mutant of Gapex-5 that lacks GEF activity, but can still interact with Rab31. No such mutant has been identified.

In contrast to the inhibitory effect of overexpressing constitutively active Rab31, the knockdown of Rab31 potentiates insulin-stimulated Glut4 translocation and glucose uptake. Previous studies have shown that Rab31 has high constitutive levels of bound GTP (Bao et al., 2002; Field et al., 2000). Here, we demonstrate that insulin decreases the GTP levels on Rab31. Thus, we propose a model (Figure 7C) in which Gapex-5 maintains Rab31 in an active state in the absence of insulin, promoting the recycling of Glut4 between the GSC and endosomes. Upon the translocation of the CIP4/Gapex-5 complex to the plasma membrane in response to insulin, the local Rab31 GAP/GEF ratio in the TGN and endosomes is increased. This results in decreased activity of Rab31, permitting Glut4 to escape from the futile intracellular cycle and traffic to the plasma membrane, where Glut4 docks and fuses to transport glucose into the cell. This hypothesis will require further experimentation.

Experimental procedures

Antibodies

The anti-HA, anti-myc and anti-CblΔantibodies were purchased from Santa Cruz Biotechnology. The anti-FLAG M2 antibody was purchased from Sigma. The anti-Gapex-5 polyclonal antibody was raised against the amino-terminal residues 1–17 and was purified using protein ImmunoPure (A) IgG purification kit (Pierce). Polyclonal antibody against Caveolin and monoclonal antibodies against CIP4, EEA1, Syntaxin-6 and Rabaptin-5 were purchased from BD Biosciences and a monoclonal antibody against Rab31 was from Abnova. Phospo-Akt (ser 473) antibody was from Cell Signaling Technology. Anti-Phosphotyrosine 4G10 antibody was from Upstate Cell Signaling Solutions.

Expression Constructs

To create mammalian expression constructs of Gapex-5, the internal Eco*RI* site was destroyed by a silent mutation. The gene was now cloned into Xho*I* and Eco*RI* sites of a modified pKH3 (HA-tag) vector or pRK5myc (myc tag) vector. Myc-tagged CIP4 mutants have been previously described (Chang et al., 2002). GFP-tagged CIP4 mutants were made by subcloning into pEGFP-C1 vector. Myc-Rab22a and GST-EEA1/NT were provided by Philip Stahl (Washington University, St. Louis). Rab31 cDNA was from Francis Barr (Max Planck Institute of Biochemistry, Martinsried, Germany), and the cDNA for Rab5a was obtained from the UMR cDNA Resource Center (cdna.org). Both of these Rabs were subcloned into pKH3 or pGEX 4T-1 plasmids. Point mutants of Rab31 were created using the QuikChange method (Stratagene). The C-terminal Rab binding domain of EEA1 (residues 1245–1411) was cloned from the 3T3-L1 cDNA library by PCR and subcloned into pGEX 4T-1. The VPS9 domain and the tandem helical bundle domain of Gapex-5 (residues 1111–1437) were cloned into pGEX 4T-1. Myc-Glut1-GFP construct was from Brent Reed (Louisiana State University, Shreveport, LA). All constructs were sequenced to confirm reading frame.

Cloning of Gapex-5

To search for CIP4-interacting protein, a yeast two-hybrid cDNA library derived from 3T3-L1 adipocytes was screened essentially as described (Chang et al., 2002). The Gapex-5 yeast two-hybrid hit consisted of base pairs 1311–2916. The sequence of the cDNA fragment isolated in the yeast two-hybrid screen was used to search the NCBI database for overlapping ESTs. Alignment of the ESTs indicated a full-length sequence containing Kozak consensus box and a stop codon. The entire open reading frame was cloned by RT-PCR using total RNA from 3T3-L1 adipocytes.

Cell culture and electroporation of 3T3-L1 adipocytes

The 3T3-L1 fibroblasts were grown in DMEM containing 10% fetal bovine serum (FBS) and differentiated into adipocytes as previously described (Baumann et al., 2000). The adipocytes were transfected with Stealth™ siRNA duplexes (Invitrogen) by electroporation as previously described (Inoue et al., 2006).

Immunofluorescence Microscopy

To detect myc-tagged CIP4 or Gapex-5, the cells were stained with anti-myc polyclonal antibody (Santa Cruz Biotechnology) at 2 μg/ml. To detect HA-Gapex-5 or HA-Rab31, the cells were stained with anti-hemagglutinin (HA) mAb at 2 μg/ml. After incubation with primary antibodies, cells were incubated with Alexa^{488} or Alexa^{594} goat anti-mouse or anti-rabbit IgG at 2 μg/ml (Invitrogen). Coverslips were mounted in Vectashield mounting media (Vector Laboratories). Images were captured by using an Olympus FluoView 500 laser scanning confocal microscope.

In vitro GST pull-down assays

GST fusion proteins were expressed in BL21(DE3)pLysS *Escherichia coli* strain and purified as described (Smith and Johnson, 1988). GST pull-down assays were performed as previously described (Chang et al, 2002).

Nucleotide loading of GTPases

To nucleotide load Rab proteins overexpressed in Cos-1 cells, the cells were lysed using a buffer containing 20 mM Hepes [pH 7.5], 150 mM NaCl, 5 mM EDTA, 5% glycerol and 1% NP-40. The lysates were combined with an equal volume of the same buffer lacking NP-40 and incubated with 1 mM GTPγS or 2 mM GDP at 30°C for 30 min. The reaction was stopped by adding 60 mM $MgCl_2$. GST-tagged Rab proteins were similarly loaded with nucleotides using the lysis buffer containing 0.5% NP-40.

2-Deoxyglucose uptake assay

The RNAi-transfected cells were re-seeded on 12-well plates and cultured for 3 days. The uptake of 2-deoxyglucose was measured as previously described (Inoue et al., 2006).

Establishment of adipocytes stably expressing myc-Glut4-eGFP

3T3-L1 fibroblasts were infected with retroviral particles containing myc-Glut4-eGFP (Bogan et al., 2001), and myc-Glut4-eGFP expressing cells were sorted by FACS based on the FITC intensity. Cells with high expression levels of Glut4-eGFP were collected and differentiated into adipocytes.

Rab31 activation assay

To determine the effect of insulin on the activation state of Rab31, 3T3-L1 adipocytes were serum starved for 3 hr and then stimulated with or without insulin and lysed using Rab31

activation assay lysis buffer (20 mM Hepes [pH 7.5], 100 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 5% glycerol and 1% Triton-X-100), supplemented with protease inhibitor cocktail (Complete Mini, EDTA-free) from Roche. Lysates were diluted with an equal volume of the lysis buffer lacking Triton-X-100 and incubated with 15 μg of GST-EEAI/NT at 4°C rocking. After 1 hr, the beads were washed 4 times using the lysis buffer (containing Triton-X-100). The pull-downs and lysates were subjected to electrophoresis and analyzed by immunoblotting using an anti-Rab31 antibody.

Accession numbers

The sequence of mouse Gapex-5 has been deposited into GenBank under accession no. EF155419.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank members of the Saltiel lab for critical reading of the manuscript and helpful discussions. We acknowledge Dr. Mei Zhang for expert technical assistance in cloning the full-length Gapex-5. We also acknowledge the technical assistance of Emily Lampe. This work utilized the Morphology and Image Analysis Core of the Michigan Diabetes Research and Training Center funded by NIH5P60-DK20572 from the National Institute of Diabetes & Digestive & Kidney Diseases. This work was supported by NIH grants RO1-DK061618 to ARS and DK59291 to JEP.

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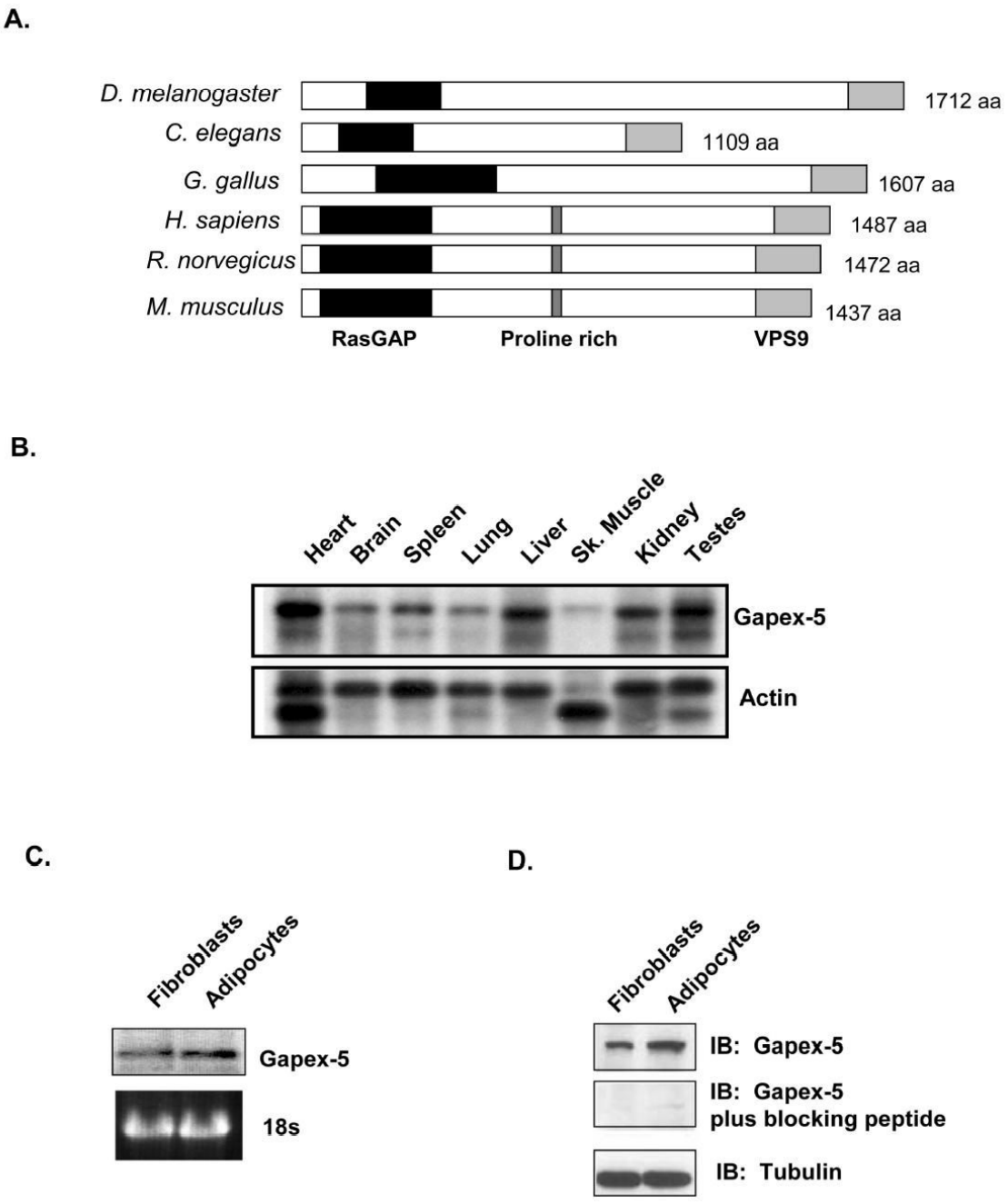

Figure 1. Gapex-5 domain structure and expression profile

A) Gapex-5 is an evolutionarily conserved protein that contains an N-terminal RasGAP domain and a C-terminal VPS9 domain, which is a Rab5 GDP/GTP exchange factor. The mammalian protein also has a central proline-rich sequence. B) Multi-tissue Northern blot analysis of Gapex-5. C) Northern blot analysis of Gapex-5 expression in 3T3-L1 fibroblasts and fully differentiated adipocytes. D) Western blot analysis of Gapex-5 expression in fibroblasts and adipocytes using a polyclonal anti-Gapex-5 antibody. The specificity of the antibody was determined using a blocking peptide.

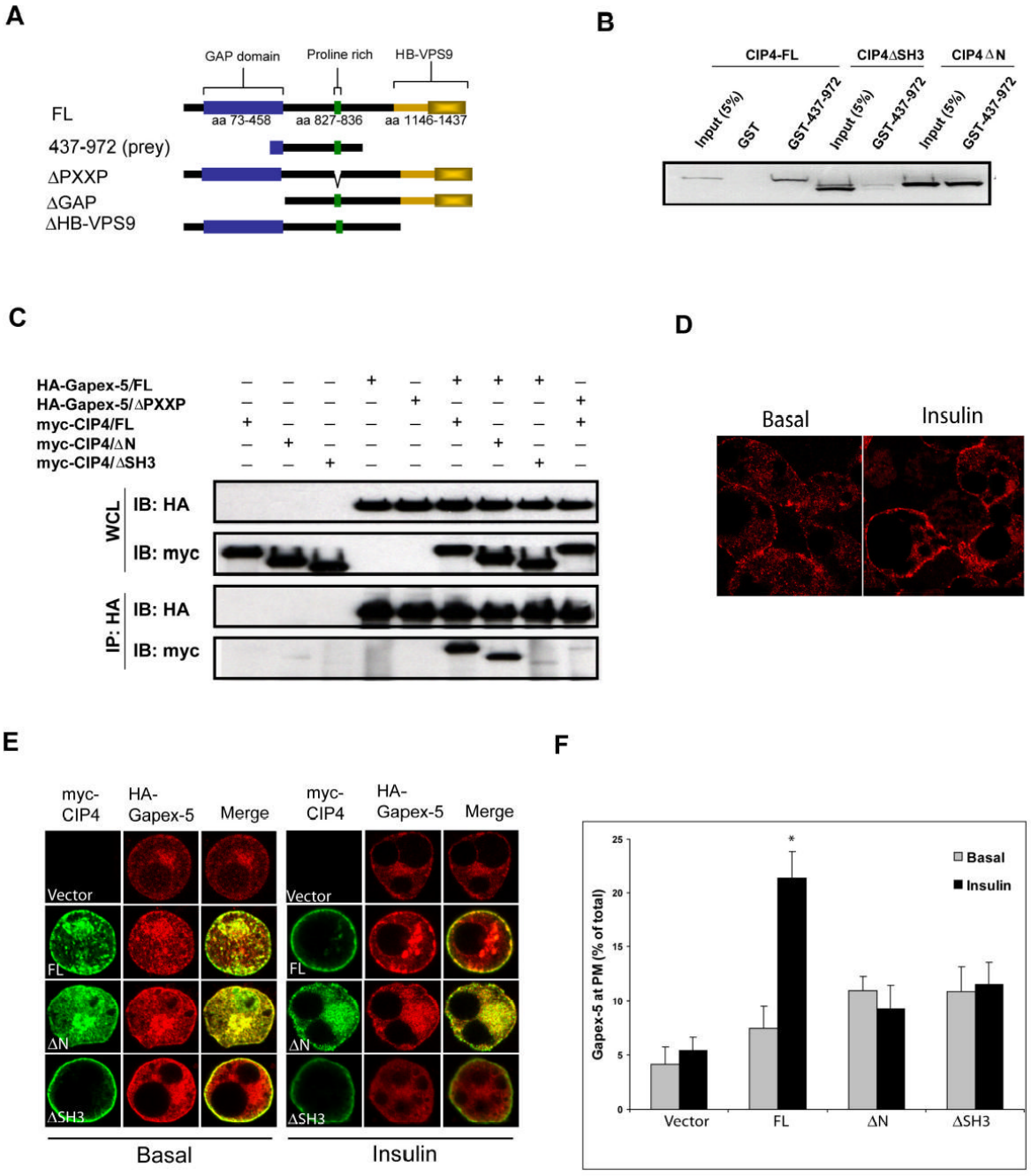

Figure 2. Interaction of Gapex-5 with CIP4

A) Schematic of Gapex-5 mutants used in this study. B) GST-tagged Gapex-5 fragment consisting of aa 437–972 was used to pull down myc-tagged CIP4 mutants overexpressed in Cos-1 cells. C) Cos-1 cells were transiently transfected with HA-Gapex-5 and/or myc-CIP4 constructs as indicated. Whole cell lysates (upper panels) or anti-HA immunoprecipitates were subjected to immunoblotting with anti-myc or anti-HA antibodies. D) 3T3-L1 adipocytes were serum starved for 3 hr and then stimulated with or without 100 nM insulin. The cells were fixed and immuno-stained using a monoclonal antibody against CIP4. E) 3T3-L1 adipocytes were electroporated with myc-CIP4 and/or HA-Gapex-5 as indicated. After 24 hr, cells were serum starved for 3 hr and then stimulated with or without 100 nM insulin. The cells were fixed,

immuno-stained and analyzed by confocal microscopy. Representative images are shown from 4 independent experiments. F) Adipocytes were transfected with HA-Gapex-5 alone (Vector) or together with myc-CIP4 mutants as described in panel 2E. The amount of HA-Gapex-5 localized at the plasma membrane as a percentage of total HA-Gapex-5 was quantified from at least 10 cells per condition per experiment using the NIH ImageJ program. The signal intensity at the plasma membrane was expressed as a percentage of total signal intensity (intracellular plus plasma membrane, arbitrary units). To ensure that the changes did not simply reflect differences in area, plasma membrane area to total cell area was measured. The data are presented as mean ± SD and is representative of two separate experiments. *Significant difference, p-value$<10^{-7}$ vs. Vector (insulin).

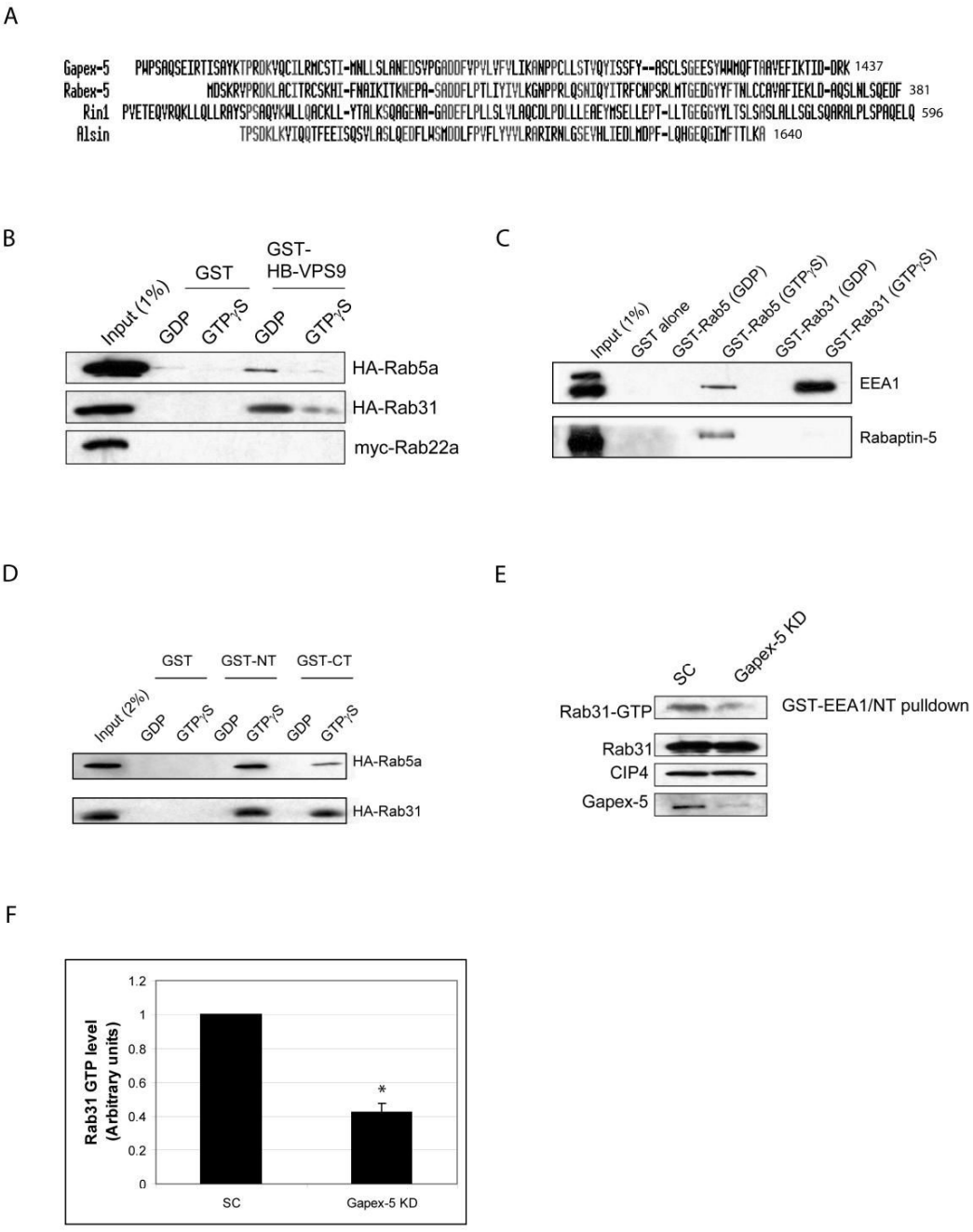

Figure 3. Identification of Gapex-5 as a Rab GEF and EEA1 as an effector of Rab31

A) Alignment of the VPS9 domain of Gapex-5 with the VPS9 domains of known Rab5 GEFs, Rabex-5, Rin1 and Alsin. B) Lysates from Cos-1 cells overexpressing HA-Rab5a, HA-Rab31 or myc-Rab22a were incubated with GDP or GTPγS. Following pulldown with GST-tagged helical bundle/VPS9 domains of Gapex-5 (GST-HB-VPS9), the Rab proteins were detected by immunoblotting using anti-HA or anti-myc antibodies as indicated. C) GST-tagged Rab5 or Rab31 were loaded with GDP or GTPγS and incubated with lysates from Cos-1 cells. Endogenous EEA1 and Rabaptin-5 were detected by immunoblotting. D) HA-Rab5a or Rab31 overexpressed in Cos-1 cells were loaded with nucleotides and pulled down using GST-tagged N- or C-terminal Rab binding domain of EEA1 (GST-NT or GST-CT, respectively). E)

HEK-293 cells were transfected with an siRNA oligonucleotide for human Gapex-5 (Gapex-5 KD) or a matching scrambled siRNA (SC). Forty eight hours later, the cells were transfected with HA-Rab31. After another 24 hrs, the cells were harvested and used in pulldown with GST-EEA1/NT. Rab31 was detected using an anti-HA antibody. Gapex-5 and CIP4 were detected using their respective antibodies. F). Rab31-GTP levels normalized to total Rab31 were quantified using the ImageJ program. The data are presented as mean ± SD of three independent experiments. *Significant difference, p-value=0.003.

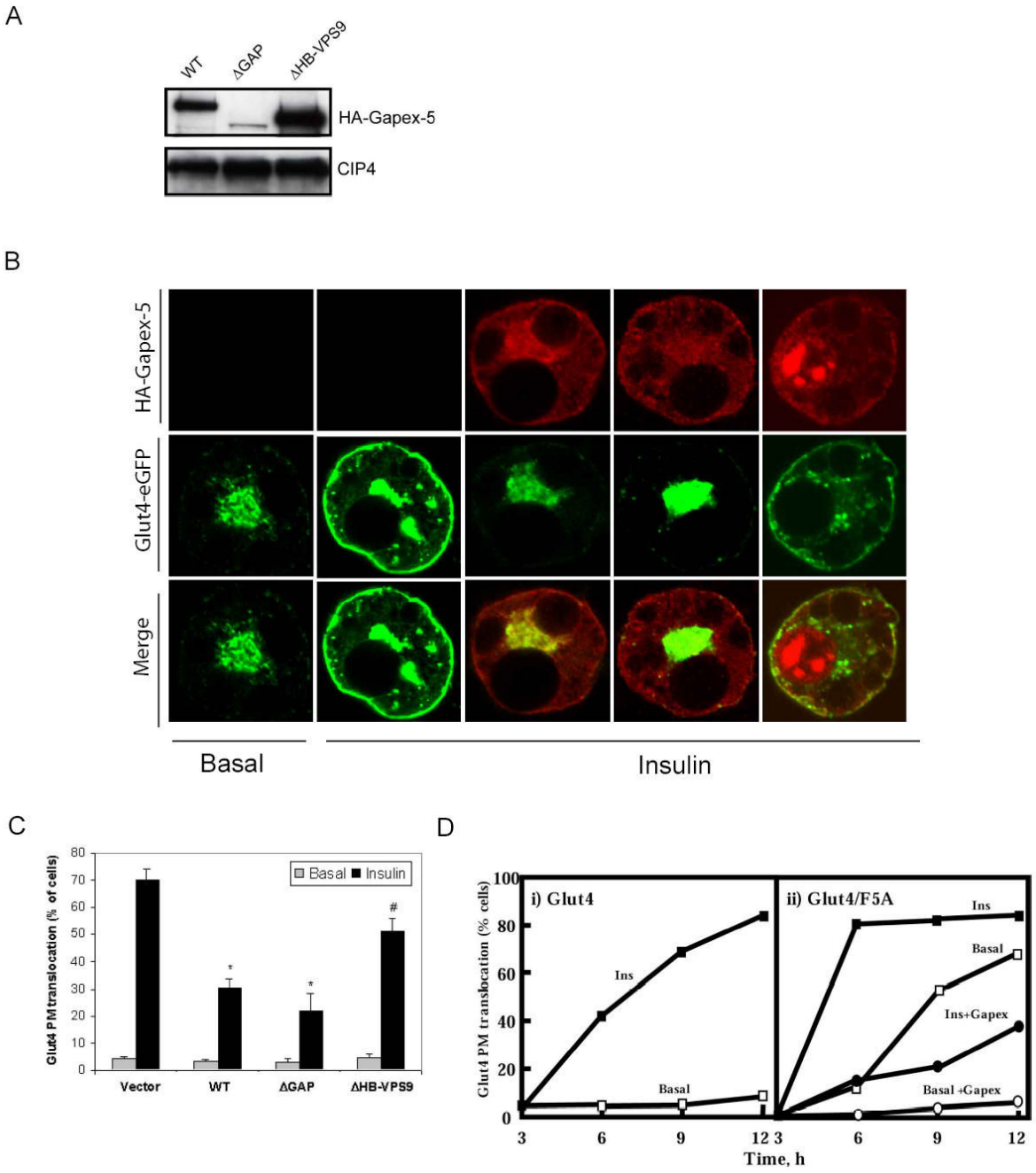

Figure 4. Gapex-5 blocks insulin-stimulated Glut4 translocation in 3T3-L1 adipocytes

A) Expression of HA-tagged Gapex-5 constructs in 3T3L1 adipocytes. Cell lysates prepared from 3T3-L1 adipocytes electroporated with full length (FL), ΔGAP or ΔHB–VPS9 constructs of HA-Gapex-5 were resolved by SDS-PAGE and immunoblotted using an anti-HA antibody. CIP4 was detected with a polyclonal anti-CIP4 antibody as a loading control. B) 3T3-L1 adipocytes were transfected with Glut4-eGFP alone (vector) or in combination with HA-tagged Gapex-5. After 24 hr, the cells were serum starved and then stimulated with or without 100 nM insulin for 30 min. The cells were fixed and then stained using a monoclonal anti-HA antibody. C) Quantification of Glut4 translocation. The cells with GFP rim staining were marked as positive for Glut4 translocation. At least 50 cells per condition per experiment were

counted. The data are mean ± SD of 5 experiments. *Significant difference vs. vector (insulin), p-value ≤0.025; #Significant difference vs. WT (insulin), p-value=0.03. D) 3T3-L1 adipocytes were transfected with Glut4-eGFP/F5A alone or together with HA-Gapex-5. At 3, 6, 9 or 12 hr post-transfection, cells were serum starved for 3 hr and then stimulated with or without insulin. Plasma membrane translocation of Glut4 was quantified by determining the percentage of cells with GFP rim staining. At each time point, 50 cells per condition were counted. The data are an average of two separate experiments. The translocation of wild-type Glut4-eGFP from a representative experiment is shown for comparison.

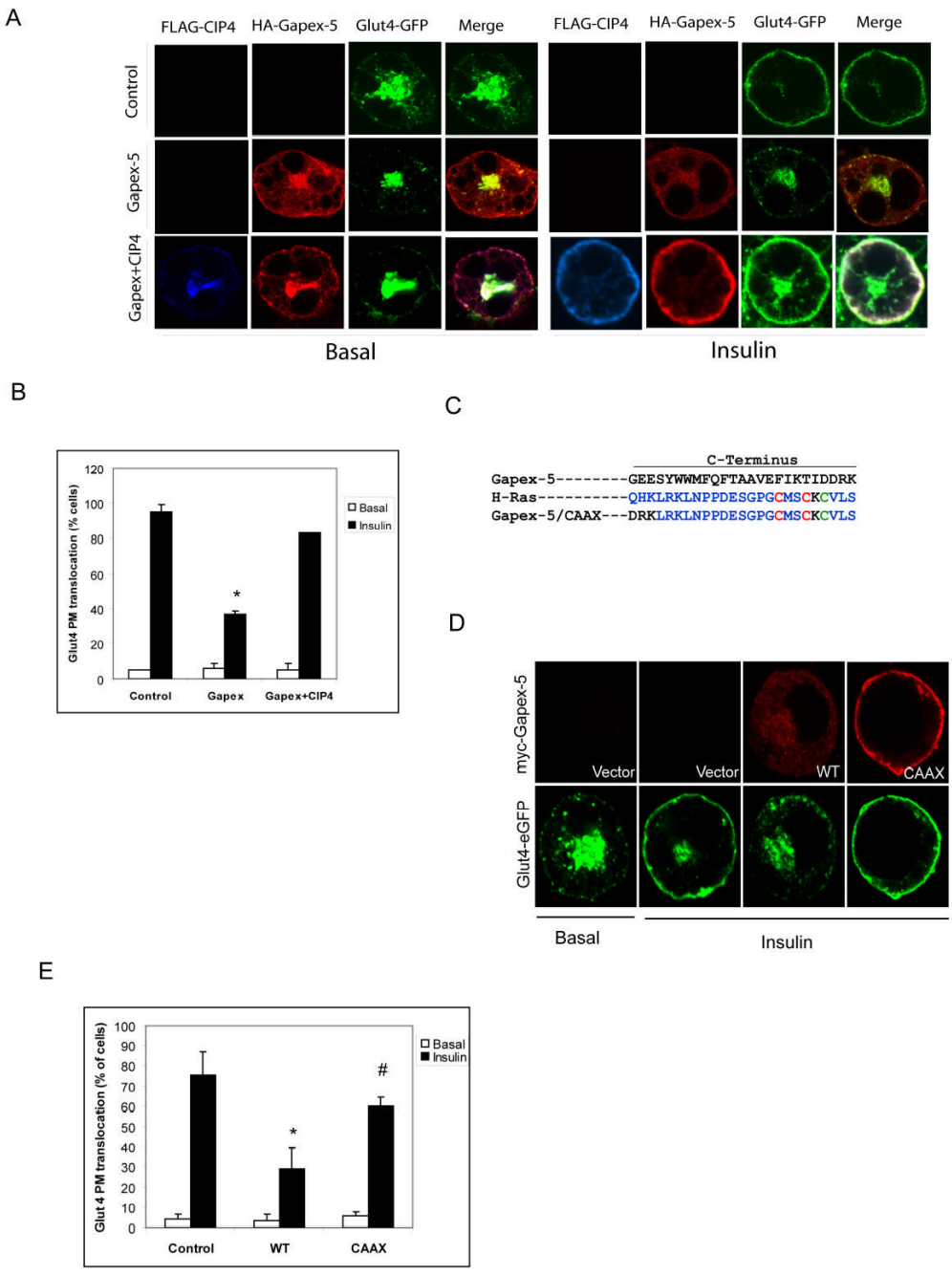

Figure 5. Plasma membrane targeting of Gapex-5 relieves its inhibitory effect on Glut4 translocation

A) 3T3-L1 adipocytes were transfected with Glut4-eGFP alone (control), Glut4-eGFP and HA-Gapex-5, or Glut4-eGFP, HA-Gapex-5 and FLAG-CIP4. After 24 hr, the cells were serum starved for 3 hr and then stimulated with or without 100 nM insulin for 30 min. The cells were fixed with paraformaldehyde and then immuno-stained using anti-HA polyclonal and anti-FLAG monoclonal antibodies. B) Quantification of the Glut4 translocation. One hundred cells per condition were counted. The data are mean ± SD of 2 separate experiments. *Significant difference vs. control (insulin), p-value=0.002. C) Schematic of Gapex-5/CAAX chimera. D) 3T3-L1 adipocytes were transfected with Glut4-eGFP alone or together with myc-Gapex-5

wild-type or its CAAX chimera. The cells were fixed and immuno-stained and analyzed by confocal microcopy. Representative images are shown. E) Quantification of Glut4 translocation. The data are mean ± SD of 3-5 separate experiments. *Significant difference vs. Control (insulin), p-value<0.03; #significant difference vs. WT (insulin), p-value<0.001.

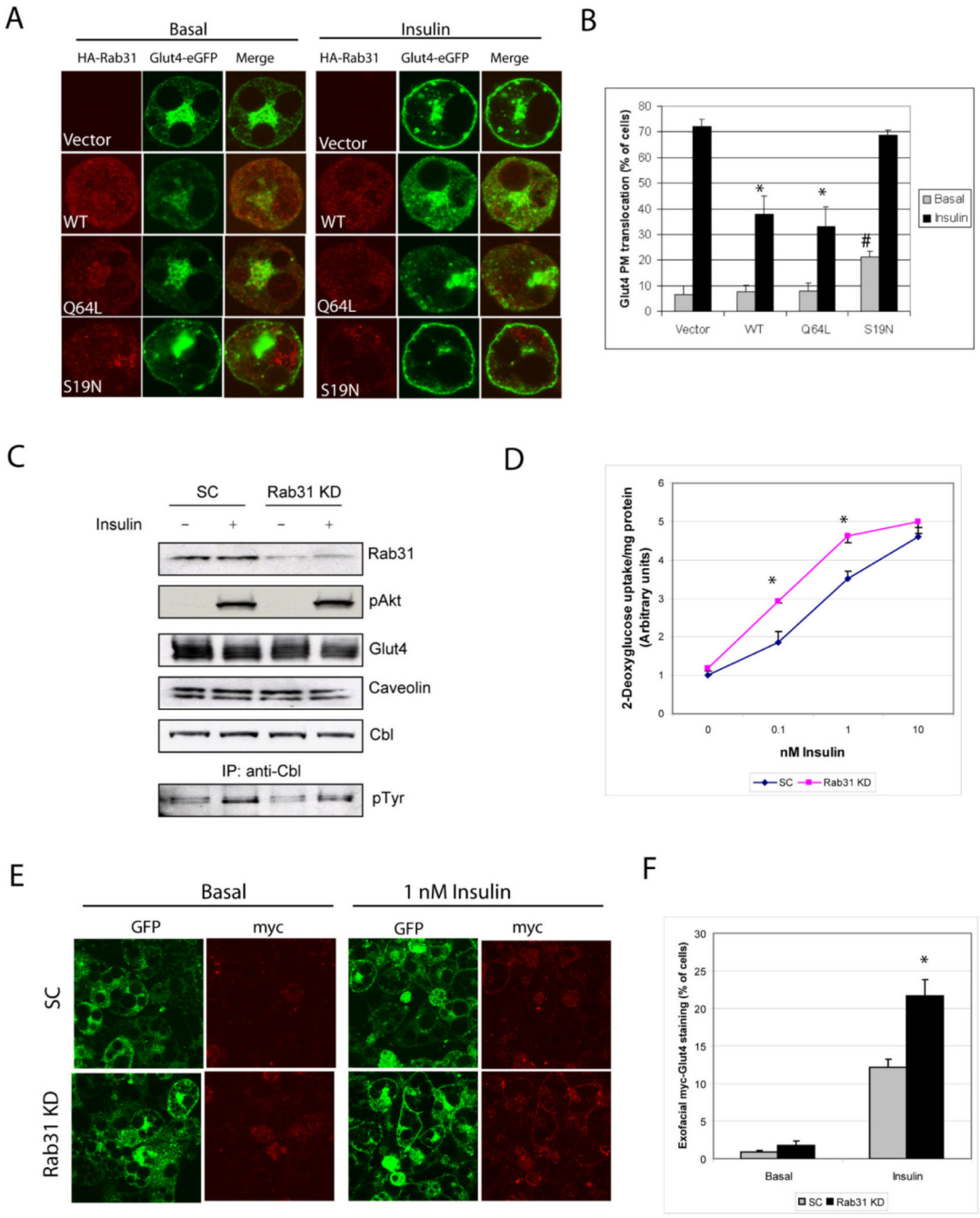

Figure 6. Effect of Rab31 on Glut4 translocation and glucose uptake

A) 3T3-L1 adipocytes were transfected with Glut4-eGFP alone (vector) or together with HA-tagged Rab31. The cells were fixed, immuno-stained and analyzed by confocal microscopy. Representative images are shown. B) Glut4 translocation was quantified by determining the percentage of cells with GFP rim staining. At least 50 cells per condition were counted. The data are mean ± SD of 3 separate experiments. *Significant difference vs. Vector (insulin), p-value<0.02; #significant difference vs. Vector (basal), p-value=0.03. C) 3T3-L1 adipocytes were electroporated with siRNA for Rab31 (Rab31 KD) or a matching scrambled control siRNA (SC). After 72 hr, the cells were serum starved for 3 hr and then stimulated with or without 100 nM insulin. Lysates were resolved by SDS-PAGE and subjected to

immunoblotting using anti-Rab31, phospho-Akt, Glut4, Caveolin and Cbl antibodies. The phosphorylation of Cbl immunoprecipitated from control and Rab31 knockdown lysates was detected by immunoblotting with phosphotyrosine 4G10 antibody. D) At 72 hr after transfection with Rab31 knockdown siRNA (Rab31 KD) or a matching scrambled oligonucleotide (SC), adipocytes were serum starved for 3 hr and then stimulated with 0, 0.1, 1, or 10 nM insulin. The uptake of 2-deoxyglucose was measured as described under Experimental Procedures. The data are mean ± SD of triplicate determinations and were reproduced five times. *Significant difference, p-value $\leq$ 0.025. E) Adipocytes stably infected with retrovirus expressing myc-Glut4-eGFP reporter were transfected with Rab31 knockdown or scrambled siRNA. After 72 hr, the cells were serum-starved and stimulated with or without 1 nM insulin. The cells were fixed and then immuno-stained using an anti-myc polyclonal antibody. Representative images are shown from 3 independent experiments. F) Quantification of Glut4 translocation to the plasma membrane. At least 300 cells per condition per experiment were scored for exofacial myc-Glut4 staining in two separate experiments. Data are presented as mean ± SEM. *Significant difference vs. SC (insulin), p-value<0.02.

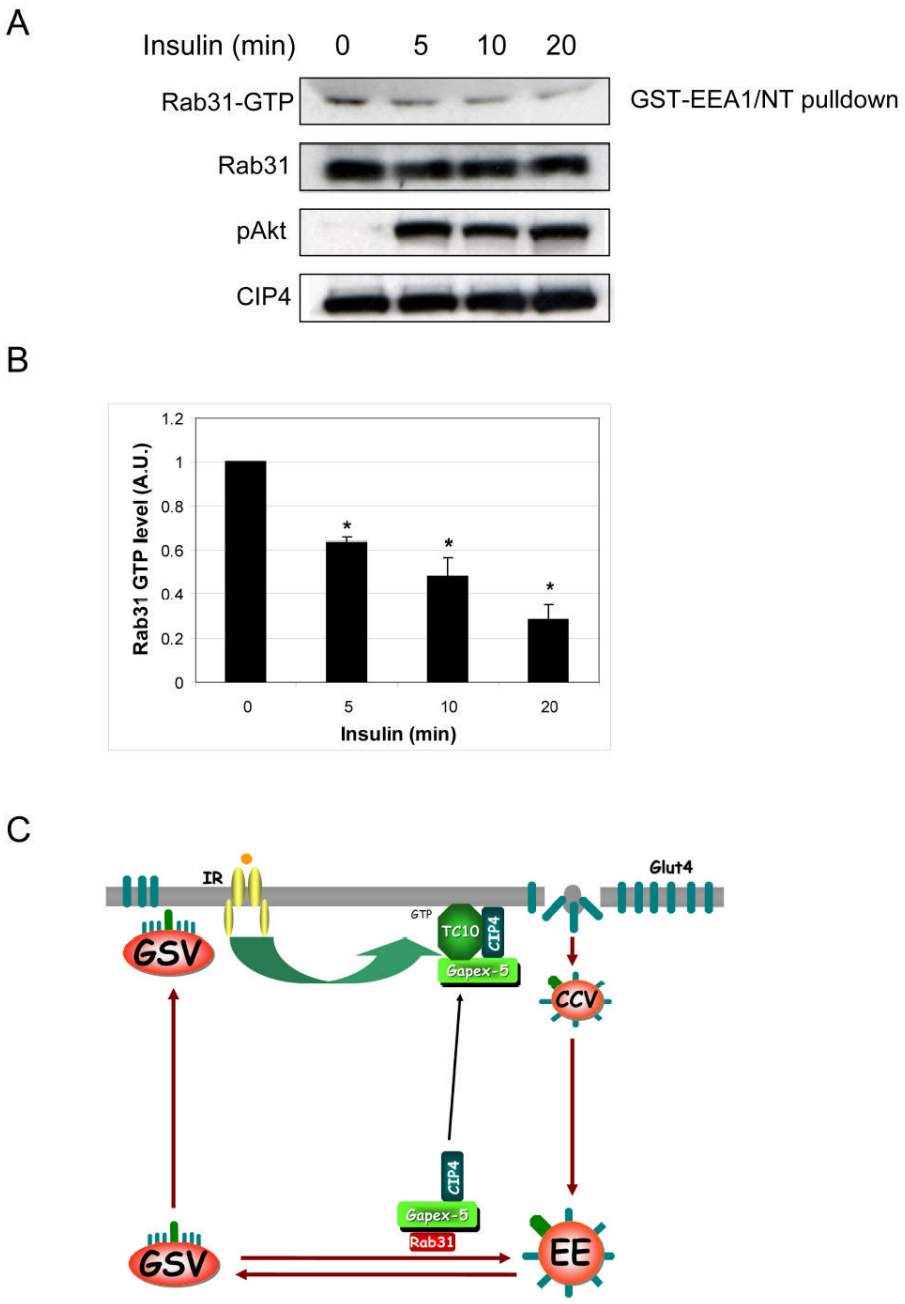

Figure 7. Insulin inhibits the activity of Rab31 in 3T3-L1 adipocytes

A) 3T3-L1 adipocytes were stimulated with 100 nM insulin for 0, 5, 10 or 20 min. The activation state of endogenous Rab31 was determined using GST-EEA1/NT as described in Experimental Procedures. B) Rab31-GTP levels normalized to total Rab31 were quantified using the ImageJ program. The data are presented as mean ± SD of three independent experiments. *Significant difference, p-value<0.03. C) A hypothetical model describing how Gapex-5 and Rab31 regulate Glut4 translocation. In the absence of insulin, Glut4 is rapidly internalized through clathrin-coated vesicles (CCV) and retained intracellularly by cycling between specialized insulin-responsive Glut4 storage vesicles (GSV) and early endosomes (EE). Gapex-5 maintains Rab31 in an active state and promotes fusion of Glut4 vesicles with

endosomes, resulting in a futile cycle that retains Glut4 inside the cell. Insulin stimulation results in the activation of the Rho family GTPase TC10 and recruitment of the CIP4/Gapex-5 complex to the plasma membrane. This results in a decrease of Rab31 activity, permitting insulin responsive Glut4 vesicles to escape the futile intracellular cycle and translocate to the plasma membrane, where Glut4 docks and fuses to transport glucose into the cell. This hypothesis will require further experimentation.