Translocation of the insulin-regulated glucose transporter GLUT4 to the cell surface is dependent on the phosphatidylinositol 3-kinase/Akt pathway. The RabGAP (Rab GTPase-activating protein) AS160 (Akt substrate of 160 kDa) is a direct substrate of Akt and plays an essential role in the regulation of GLUT4 trafficking. We have used liquid chromatography tandem mass spectrometry to identify several 14-3-3 isoforms as substrates of Akt and plays an essential role in the regulation of the phosphatidylinositol 3-kinase/Akt pathway. The interaction of 14-3-3 with target proteins, and scaffolding. In the present study, we explored the role of 14-3-3 in insulin-stimulated GLUT4 translocation through its interaction with the RabGAP AS160.

A Role for 14-3-3 in Insulin-stimulated GLUT4 Translocation through Its Interaction with the RabGAP AS160*

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The insulin-regulated glucose transporter GLUT4 is mainly expressed in muscle and adipose tissue and plays an important role in glucose homeostasis. Its translocation from intracellular compartments to the cell surface in response to insulin is a multistep process involving intracellular sorting, vesicular transport to the cell surface along cytoskeletal elements, reorganization of cortical actin, and finally docking, priming, and fusion of so-called GLUT4 storage vesicle with the cell surface (1–4). Although several players in each step are known, the exact nodes between signaling and GLUT4 trafficking still remain elusive. The phosphatidylinositol 3-kinase/Akt pathway has been shown to play an essential role in GLUT4 translocation. One of its many substrates, the Akt substrate of 160 kDa, AS160, contains a Rab GTPase-activating domain, but its cognate in vivo Rab has not yet been identified (5, 6).

There is accumulating evidence in the recent literature that this RabGAP plays an important role in insulin-triggered GLUT4 translocation. Insulin treatment of adipocytes results in phosphorylation of AS160 at six separate sites, and this appears to be mediated by Akt (5, 6). Overexpression of an AS160 mutant, in which four of these phosphorylation sites have been mutated to alanines (AS1604P), inhibits insulin-stimulated GLUT4 translocation to the cell surface in adipocytes. This effect is dependent on an intact RabGAP domain because simultaneous disruption of the putative GAP domain in AS1604P overcame this inhibitory effect on GLUT4 translocation (6, 7). This observation indicates that the GAP activity of this protein has an important role in GLUT4 trafficking, thus providing one of the first links between signaling and membrane trafficking in this process. More recent evidence suggests that AS160 may inhibit GLUT4 trafficking under basal conditions because knockdown of AS160 using RNA interference resulted in insulin-independent translocation of GLUT4 to the cell surface (8, 9). This supports the model originally proposed by Lienhard and colleagues (6) that under basal conditions, AS160 may inhibit a Rab protein required for GLUT4 translocation and that insulin-dependent phosphorylation of AS160 overcomes this inhibitory effect. Adding another layer to this model, it has recently been shown that under basal conditions, AS160 binds to GLUT4 storage vesicles, at least in part by a direct interaction with the cytosolic tail of the vesicle cargo protein insulin-responsive amino peptidase (IRAP), and it is released into the cytosol upon insulin stimulation (9). Several Ras, including Ras 2a, 8a, 10, 11, and 14, have been found to associate with GLUT4 vesicles (9, 10). AS160 has been shown to display some GTPase activity toward Rabs 2A, 8A, 10, and 14 in vitro (10). However, studies linking these Rabs to AS160 function and GLUT4 trafficking in vivo remain to be described.

14-3-3 proteins comprise a family of seven isoforms in mammals (β, γ, ε, ζ, η, and σ) (11–14). Their major role is facilitated by interacting with phospho-serine or phospho-threonine residues in a variety of proteins, often in response to growth factor stimulation. The interaction of 14-3-3 with target proteins has been shown to encode a variety of functions including subcellular redistribution, altered protein conformation, protection from proteolysis, impaired interaction with other proteins, and scaffolding. In the present study, we
describe 14-3-3 as a novel interaction partner for AS160 and provide evidence that this interaction plays an important role in insulin-regulated GLUT4 trafficking. This adds to the list of roles ascribed to 14-3-3 proteins in biological systems.

**EXPERIMENTAL PROCEDURES**

**Materials**—FLAG-AS160_WT and FLAG-AS160_A18 constructs were a gift from Gus Lienhard (Dartmouth, NH), pGEX-GST-14-3-3 β was obtained from John Hancock (Institute of Molecular Bioscience, Brisbane, Australia), and GST fusions of 14-3-3 isoforms were kindly provided by Walter Hunziker (Institute of Molecular and Cell Biology, Singapore) (15). pCR3.1 Myr-α-14-3-3 was a gift from Gus Lienhard (Dartmouth, NH), pGEX-GST-14-3-3 (16) was obtained from John Hancock (Institute of Molecular Biosciences, Singapore) (15). pBabe-HA-GLUT4 retrovirus (16) was obtained from Howard Green, cultured, and differentiated into 3T3-L1 adipocytes and newborn calf serum were purchased from Sigma. Anti-phospho-Akt substrate, anti-phospho-Ser-473 Akt was from Cell Signaling Technologies (Danvers, MA), anti-FLAG(M2) was from Sigma, anti-HA peptide (16B12) was from Covance Research Products (Richmond, CA), horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences (Buckinghamshire, UK), and Alexa Fluor 488 and Cy3-conjugated secondary antibodies were from Molecular Probes (Leiden, the Netherlands) and Jackson ImmunoResearch (West Grove, PA). The AS160 antibodies have been described earlier (9) or were purchased from Upstate Biotechnology (Lake Placid, NY).

**Immunoprecipitation and Western Blotting**—FLAG-AS160 expressing CHO cells or 3T3-L1 adipocytes were incubated in Dulbecco’s modified Eagle’s medium for 2 h, stimulated with 100 nM insulin for 15 min or preincubated with 100 nM wortmannin for 10 min before the addition of insulin, washed twice with ice-cold phosphate-buffered saline, and lysed by 10 strokes through a 27-gauge needle in Nonidet P-40 buffer (1% Nonidet P-40, 137 mM sodium chloride, 10% glycerol, 25 mM Tris, pH 7.4) supplemented with Complete protease inhibitor mixture and phosphatase inhibitors (2 mM sodium orthovanadate, 1 mM pyrophosphate, 10 mM sodium fluoride) at 4 °C. The homogenate was centrifuged at 18,000 × g for 20 min, and the supernatant was incubated with anti-FLAG antibody and protein G beads for 2 h at 4 °C. The beads were then washed, and FLAG-AS160 was released from the antibody by incubating for 1 h with 100 μg/ml 3xFLAG peptide (Sigma). For cotransfection of GST-14-3-3 with FLAG-AS160, the lysate was incubated with glutathione-Sepharose beads, and the beads were washed and eluted with sample buffer. For GST pull downs, lysate from single transfected CHO IR/IRS-1 cells was incubated with either GST alone or GST-14-3-3 and glutathione-Sepharose beads. Beads were washed and boiled in sample buffer. SDS-PAGE and Western blotting was performed according to standard protocols. For the in vivo immunoprecipitation, 3T3-L1 adipocytes were treated as above but lysed in 1% Triton X-100/60 mM β-octylglucoside (137 mM sodium chloride, 10% glycerol, 25 mM Tris, pH 7.4, complete protease inhibitor mixture, 2 mM sodium orthovanadate, 1 mM pyrophosphate, 10 mM sodium fluoride). AS160 was immunoprecipitated from the lysate using 2 μl of AS160 antibody (Upstate Biotechnology) and protein A beads for 2 h at 4 °C.

**Mass Spectrometry**—Sypro Ruby-stained gel bands were excised, destained for 30 min in 50% acetonitrile and 500 mM ammonium bicarbonate, and dehydrated in 100% acetonitrile for 30 min. The acetonitrile was removed, and the proteins were digested with modified trypsin (12.5 ng/μl) in 100 mM NH₄HCO₃ overnight at 37 °C. Peptides were extracted by the addition of 5% formic acid for 1 h at room temperature followed by 1 volume of 100% acetonitrile using 2 μl of 5% formic acid and stored at −20 °C until analyzed. Samples were desalted and subjected to mass spectrometry (with the exception that the strong cation exchange cartridge separation was omitted), and MS/MS data were analyzed as described earlier (9).

**Immunofluorescence Microscopy**—3T3-L1 adipocytes were cultured and electroporated and seeded on glass coverslips. Prior to incubation in the absence or presence of 100 nM insulin for 15 min, cells were serum-depleted for 2 h at 37 °C. After washing in cold phosphate-buffered saline, cells were fixed with 3% paraformaldehyde (ProSciTech, Thuringowa Central, Australia) and quenched with 50 mM glycine in phosphate-buffered saline.
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saline. Cells were then blocked in phosphate-buffered saline containing 2% bovine serum albumin and labeled for surface HA-GLUT4 as described previously (9). Cells were then washed and permeabilized and labeled with the AS160 antibody to identify the electroporated cells. Primary antibodies were detected with anti-rabbit Alexa Fluor 488 and anti-mouse Cy3-conjugated secondary antibodies. Optical sections were obtained through separate scans for Alexa Fluor 488 and Cy3 using the Leica TCS SP confocal laser scanning microscope. For quantification of HA, surface staining random images were collected with the same confocal settings, and images were analyzed using the Wright Cell Imaging Facility Image J software. Five small ring-shaped regions of interest were placed at equal distances on the edge of the AS160 staining to select the plasma membrane region of cells expressing respective AS160 constructs. The rings were then copied onto the corresponding image of surface HA-GLUT4 staining, and the mean fluorescence was measured.

RESULTS

Identification of 14-3-3 as an AS160-binding Protein—The Rab GTPase-activating protein AS160 is so far the last known intermediate in the phosphatidylinositol 3-kinase-dependent signal transduction pathway leading to GLUT4 translocation. To further define the role of AS160 in insulin-regulated GLUT4 translocation and possibly find additional proteins involved in insulin-stimulated GLUT4 translocation, we sought to identify new AS160 interaction partners. CHO IR/IRS1 cells were transiently transfected with FLAG-tagged AS160. Immunoprecipitation of AS160 from basal versus insulin-treated cells revealed a 30-kDa band on SDS-PAGE that associated with AS160 in a partially insulin-dependent manner. LC-MS/MS analysis revealed that this band contained two members of the 14-3-3 family, ε and θ (Fig. 1A). Three major Sypro Ruby-stained bands were identified in the AS160 immunoprecipitation migrating at a similar position to immunoreactive AS160 (Fig. 1A, asterisks). Each of these bands was identified as AS160 by mass spectrometry, and so it is assumed that they represent degradation products or covalently modified forms of AS160. In addition, antibody IgG was detected by LC-MS/MS at 55 and 25 kDa, respectively. With the exception of 14-3-3, all other bands were below the limit of detection by LC-MS/MS. The interaction between AS160 and 14-3-3 was confirmed with two different approaches involving far Western overlay using GST-14-3-3 and affinity purification of FLAG-AS160 from CHO cells using GST-14-3-3 β as bait (Fig. 1C). In each experiment, the interaction of AS160 with 14-3-3 was increased upon insulin stimulation.

To establish whether there was any 14-3-3 isoform specificity in regard to AS160 binding, we cotransfected GST fusion proteins comprising each of the seven known mammalian 14-3-3 isoforms together with AS160 in CHO IR/IRS-1 cells and performed glutathione pull-downs (Fig. 2A). These studies indicated that all 14-3-3 isoforms interacted with AS160 to a similar extent in vivo, with the exception of the α isoform, which showed reduced binding.

We next attempted to establish the interaction between endogenous 14-3-3 and AS160 in adipocytes. Adipocyte lysates were immunoprecipitated using AS160 antibody. As indicated in Fig. 2B, there was a small amount of 14-3-3 associated with AS160 under basal conditions. However, with insulin, we observed a 4.1 ± 0.8-fold increase in the amount of 14-3-3 associated with AS160. This interaction was wortmannin-sensitive, indicating a role for Akt-dependent phosphorylation.

Akt Dependence of 14-3-3 Binding to AS160 and Mapping of the Binding Site to Threonine 642—We next set out to determine whether the 14-3-3/AS160 interaction was Akt-dependent. As shown in Fig. 3A, AS160 underwent an insulin-dependent increase in phosphorylation following expression in CHO IR/IRS1 cells as determined by immunoblotting with the phospho-Akt substrate antibody. Furthermore, we observed an insulin-dependent increase in the association of endogenous 14-3-3 with FLAG-AS160 (Fig. 3A, lanes 3 and 4). To determine whether this interaction was mediated by Akt-dependent phosphorylation, we cotransfected CHO IR/IRS-1 cells with FLAG-AS160 together with a constitutively active myristoylated Akt mutant (Fig. 3A, lanes 5 and 6). Coexpression of constitutively active Akt resulted in a marked increase in Akt phosphorylation even in cells incubated in the absence of insulin. We also observed a concomitant increase in the association of 14-3-3 with AS160, which occurred in an insulin-independent manner.

These studies are consistent with a role for Akt in the insulin-dependent association of AS160 and 14-3-3, pointing to a role for phosphorylation. We next constructed a series of AS160 point mutants (Fig. 3B) to map the 14-3-3 binding site. Point mutations were introduced at the Akt phosphorylation sites in

FIGURE 1. AS160 interacts with 14-3-3 in an insulin-dependent manner. A, CHO IR/IRS1 cells were transiently transfected with a FLAG-tagged AS160 construct or a control construct (vector) and incubated with (+) or without (−) 100 nM insulin for 15 min. After immunoprecipitation with anti-FLAG antibody, proteins were eluted from the beads with a 3xFLAG peptide, run on SDS-PAGE, and stained with Sypro Ruby protein stain. The gel was sliced, and protein bands were analyzed by LC-MS/MS. A 30-kDa band showing an insulin-dependent association was identified as 14-3-3 isoforms ε and θ (n = 2). AS160 was detected in three bands indicated by asterisks. Other visible bands apart from the IgG bands were below the detection level. B, samples prepared as in A were immunoblotted with anti-FLAG and anti-phospho-Akt substrate (pAS) antibodies or overlaid with GST-14-3-3 followed by incubation with anti-GST antibody. Shown is a representative blot of three separate experiments. IP, immunoprecipitation; WB, Western blot; C, FLAG-AS160-expressing CHO IR/IRS-1 cells were incubated in the absence or presence of 100 nM insulin for 15 min or preincubated with 100 nM wortmannin (Wtm) for 10 min followed by the addition of 100 nM insulin (Ins) for another 15 min. Lysates were obtained and incubated with GST control or GST-14-3-3, or as a control, immunoprecipitated with anti-FLAG antibody. Samples were then subjected to SDS-PAGE and immunoblotted with anti-FLAG or anti-phospho-Akt substrate antibodies. Shown is a representative blot of three separate experiments.
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The Interaction of AS160 with 14-3-3 Plays a Functional Role in GLUT4 Translocation—We next wanted to determine whether 14-3-3 plays an important role in the function of AS160 and GLUT4 trafficking. Initially, we showed that overexpression of the AS160$_{1642A}$ mutant in adipocytes inhibited insulin-stimulated GLUT4 translocation consistent with earlier studies by Lienhard and colleagues (6) (Fig. 4D). These data suggest that loss of 14-3-3 binding interferes with insulin regulation of AS160 and GLUT4 translocation. To determine the role of 14-3-3 binding to AS160 independently of other factors such as increased phosphorylation, we engineered a tandem repeat of a high affinity 14-3-3 binding sequence referred to as R18 (18) into the AS160$_{4P}$ mutant adjacent to the 642 site (AS160$_{4P,R18}$ mutant, Fig. 4B). As a control, we mutated the 14-3-3 binding site in both R18 sequences from WDLLE to WLLKK (AS160$_{4P,R18KK}$ mutant). Both of these constructs were overexpressed in 3T3-L1 adipocytes by electroporation and produced proteins of the expected size that were expressed at similar levels to the AS160$_{4P}$ mutant (Fig. 4C). As expected, the AS160$_{4P,R18}$ showed a significant increase in constitutive 14-3-3 binding, whereas AS160$_{4P,R18KK}$ binding to 14-3-3 was disrupted. To measure the effect of these mutants on GLUT4 translocation, AS160$_{WT}$, AS160$_{4P}$, AS160$_{4P,R18}$ or AS160$_{4P,R18KK}$ constructs were overexpressed in HA-GLUT4 expressing 3T3-L1 adipocytes by electroporation. Surface HA staining was used to monitor GLUT4 translocation. Consistent with previous studies (6), overexpression of the AS160$_{4P}$ mutant caused a significant inhibition of insulin-stimulated GLUT4 translocation (Fig. 4, C and D). Strikingly, introduction of the R18 peptide into AS160$_{4P}$, thus restoring 14-3-3 binding, completely overcame the inhibitory effect of AS160$_{4P}$ on insulin-dependent GLUT4 translocation. Disruption of 14-3-3 binding in the AS160$_{4P,R18KK}$ mutant had a similar effect on GLUT4 translocation as the AS160$_{4P}$ mutant. These data suggest that one of the important functions of AS160 Akt phosphorylation is 14-3-3 binding. However, it is noteworthy that we have introduced the R18 peptide at a site slightly downstream of the natural 14-3-3 binding site at Thr-642. Therefore, we must entertain the possibility that this may result in a 14-3-3-mediated modification that is distinct from that mediated via interaction at Thr-642, although in view of the functional data obtained,

![Image](image.jpg)
this seems unlikely. Notably, overexpression of the AS1604P-R18 mutants in adipocytes was found to be without effect on cell surface GLUT4 levels in the absence of insulin. This illustrates that these mutants are unable to displace the function of endogenous AS160 in the basal state. However, in the presence of insulin, the AS1604P and AS1604P-R18KK mutants may act as dominant negative mutations preventing GLUT4 translocation. This argues in favor of an important role for non-phosphorylated AS160 in retaining the intracellular pool of GLUT4.

To establish whether the AS1604P-R18 mutant was still able to interact with the GLUT4 vesicle protein IRAP (9), we expressed AS160 mutants in CHO IR/IRS-1 cells and probed their binding to GST-IRAP in vitro (Fig. 4E). All three AS160 mutants showed the same degree of binding to a longer form containing the first 58 amino acids of IRAP as AS160WT. However, none of the mutants interacted with a shorter form of the IRAP cytosolic tail containing only 28 amino acids. In addition, interaction of the AS1604P-R18 mutant with IRAP was not disrupted by the
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inclusion of recombinant 14-3-3 at a concentration of 1 μM \( (K_d \text{ value for R18 peptide for 14-3-3, 70 nM (18), not shown}) \). The uninterrupted binding of AS160 \(_{4P,R18}\) with GST-IRAP suggests that the interaction of 14-3-3 is able to overcome the effects of the 4P mutant on GLUT4 translocation independently of the association of AS160 with GLUT4 vesicles. Collectively, our data show that 14-3-3 plays an important role in insulin-mediated GLUT4 translocation via its molecular interaction with AS160.

**DISCUSSION**

In the present study, we have shown that AS160 interacts with 14-3-3 in an insulin- and Akt-dependent manner and that this interaction plays an important role in insulin-regulated GLUT4 translocation. Most notably, we observed that introduction of a constitutive 14-3-3 binding site into AS160 overcame the inhibitory effects of overexpressing an AS160 Akt phosphorylation mutant on insulin action in adipocytes. These results suggest that the function of AS160 is closely linked to that of 14-3-3.

An emerging picture in the literature is that GTPase-activating proteins do not simply play a passive role in switching off GTPases following their activation, but rather, they are intimately involved in the regulatory mechanism that controls their cognate GTPases (19–21). In several other cases, this regulatory switch involves phosphorylation of the GTPase-activating protein, and indeed, 14-3-3 binding has been implicated in some instances (22–24). Most notably, Akt-dependent phosphorylation of the tuberin/hamartin complex (TSC1/TSC2) facilitates 14-3-3 binding (25–27). Members of the regulator of G protein signaling protein family (RGS), such as RGS3 and -7, have also been shown to bind to 14-3-3; however, the role of this interaction in regulating the GAP activity has been controversial (28, 29).

Previous studies by Lienhard and colleagues (6) have clearly shown an important role for Akt-mediated phosphorylation of AS160 in GLUT4 trafficking. Expression of an AS160 mutant in adipocytes, in which all four Akt phosphorylation sites were mutated to Ala, resulted in a significant impairment of insulin-dependent GLUT4 translocation (6), and this result has been verified in other studies including the present study (7, 8). We have been able to overcome the inhibitory effect achieved with this mutant by engineering a constitutive 14-3-3 binding site into the AS160 \(_{4P}\) mutant. This is not simply due to displacement of AS160 from the GLUT4 vesicle cargo protein IRAP as the AS160-IRAP interaction was maintained even in the constitutive 14-3-3 binding mutant AS160 \(_{4P,R18}\). Collectively, these data suggest that Akt-mediated phosphorylation of AS160 and in turn 14-3-3 binding play an essential role in GLUT4 trafficking. The mechanism of AS160 regulation through Akt-mediated 14-3-3 binding may involve release of AS160 from the GLUT4 storage vesicles into the cytosol, prevention of AS160 dephosphorylation and its possible consequent reassociation with GLUT4 storage vesicles, inhibition of the GAP activity of AS160, or prevention of AS160 degradation. Although we cannot exclude the possibility of a role for 14-3-3 binding in regulating the stability of AS160, such a mechanism is unlikely to regulate the rapid release of GLUT4 to the plasma membrane that is observed upon insulin stimulation. A more likely interpretation is that 14-3-3 binding may have a role in regulating the GAP activity of AS160. This may explain why the constitutive 14-3-3 binding peptide R18 was able to reverse the inhibitory effect of the 4P mutant when the 4P-R18 construct was overexpressed in adipocytes. In support of this argument, Lienhard and colleagues (6) demonstrated that the GAP activity of AS160 is an essential feature of its role in insulin-regulated GLUT4 trafficking as disrupting the GAP domain in the 4P mutant (4P-R/K) overcame its inhibitory effects on GLUT4 trafficking. The insulin-dependent binding of 14-3-3 to AS160 could also play an indirect role in the release of AS160 from GLUT4 vesicles (9). This mechanism could also lead to activation of the Rab that controls GLUT4 exocytosis. Identification of the relevant Rab protein will facilitate the distinction between these various models.

Our model predicts that there is one single 14-3-3 binding site in AS160 that is facilitated by interaction at Thr-642. We can, however, not exclude the possibility of an additional 14-3-3 binding site. Notably, we did observe significant binding of 14-3-3 to AS160 under basal conditions in both adipocytes and CHO cells (Figs. 1 and 2). Multiple 14-3-3 binding sites have also been observed in other signaling molecules such as the Ser/Thr kinase Raf (14, 30). Here 14-3-3 has been shown to play a multilayered role in modulating Raf function. Regardless, the sequence surrounding the 642 site in AS160 (RRRAHpTF-SSHPP), identified here as the major 14-3-3 binding site, fulfills most of the properties of a mode 1 14-3-3 binding site (31).

Most of the residues surrounding pT642 are in accordance with the phospho-serine-oriented degenerate peptide library screen with human 14-3-3 tau by Yaffe et al. (31) with the exception that a proline in the +2 position is absent from the AS160 sequence. (Arg preferred in −3, Phe > Arg ∼ Ser ∼ Ala in −2, Arg > Tyr ∼ Phe ∼ His in −1, and Tyr > Trp ∼ Phe ∼ His in +1.) Other 14-3-3 binding phospho-peptides lacking the proline in the +2 position have been identified, such as the Ser-939 (RARSTpSLNERP) and the Ser-981 (RCRSIpSVSEHV) sites in TSC2 (32). The R18 sequence employed in the current study mimics a phosphorylated peptide sequence, and its binding to 14-3-3 occurs in a constitutive insulin-independent manner (18).

The discovery of AS160 by Lienhard and colleagues (6) represented a major advance in the GLUT4 trafficking field as this protein provides a further critical link between signaling downstream of Akt and trafficking. The present identification of 14-3-3 as a regulated binding partner of AS160 now places this protein as a central player in the function of AS160. Although future studies are required to establish whether the interaction of 14-3-3 with AS160 blocks its GAP activity, this can only be achieved once the relevant Rab protein has been identified. Establishing the link between this effect and the regulated association of AS160 with GLUT4 storage vesicles remains another issue that requires further evaluation. Finally, in view of the dimensions of the mammalian RabGAP family comprising as many as 50 members (33, 34), one wonders whether similar mechanisms to that observed for AS160 have been duplicated in some of these other proteins to achieve similar outcomes for different vesicle transport pathways.
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