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Insulin promotes Rip11 accumulation at the plasma membrane by inhibiting a dynamin- and PI3-kinase-dependent, but Akt-independent, internalisation event

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ABSTRACT

Rip11 is a Rab11 effector protein that has been shown to be important in controlling the trafficking of several intracellular cargoes, including the fatty acid transporter FAT/CD36, V-ATPase and the glucose transporter GLUT4. We have previously demonstrated that Rip11 translocates to the plasma membrane in response to insulin and here we examine the basis of this regulated phenomenon in more detail. We show that Rip11 rapidly recycles between the cell interior and surface, and that the ability of insulin to increase the appearance of Rip11 at the cell surface involves an inhibition of Rip11 internalisation from the plasma membrane. By contrast the hormone has no effect on the rate of Rip11 translocation towards the plasma membrane. The ability of insulin to inhibit Rip11 internalisation requires dynamin and class I PI3-kinases, but is independent of the activation of the protein kinase Akt; characteristics which are very similar to the mechanism by which insulin inhibits GLUT4 endocytosis.

1. Introduction

The Rab family of small GTPases controls a multitude of trafficking pathways in eukaryotic cells [1,2]. They cycle between an inactive GDP-bound state and an active GTP-bound state, in which they interact with different specialised protein partners that can dictate their subcellular localisation or even direct their function(s) [3]. Among the Rab protein family, several Rabs have been shown to regulate trafficking steps of the endosomal pathway. For instance, Rab5 and Rab7 are involved in the endocytic trafficking of cargoes and sorting through the early-to-late endosomes and lysosomes [4–6], Rab4 is involved in the direct recycling of the transferrin receptor (TfnR) from the early/sorting endosome [7], and Rab11 is involved in its indirect recycling from the juxtanuclear recycling compartment back to the plasma membrane [8].

Several Rab11-interacting proteins have been identified on the basis of the presence of a conserved Rab11-binding domain (RBD) [9–12]. This family of proteins has been termed the Rab11 family of interacting proteins (Rab11–FIPs). Among this family, the class I Rab11–FIPs consist of Rab11–FIP1C (RCP, Rab coupling protein), Rab11–FIP2, and Rab11–FIP5 (Gaf1, pp75 or Rip11 as used in this study). They are predominantly localised to the endosomal recycling compartment, and are characterised by the presence of an N-terminal C2 domain and a C-terminal coiled-coil region encompassing the RBD. It has been shown that this coiled-coil region mediates dimerisation of the Rab11–FIPs [13], and their N-terminal C2 domain has been shown to bind to phosphoinositides (PtdIns) [14].

Among the class I Rab11–FIPs, the role of Rip11 in the recycling of transferrin has been well characterised. There is evidence that Rip11 functions in trafficking from early endosomes to juxtanuclear recycling endosomes (as suggested in [15]) and in the trafficking of cargo between the endosomal recycling compartment and the plasma membrane (as suggested in [14,16]). The latter proposal is consistent with the reported role of Rip11 in the polarised transport of proteins from apical endosomes to the apical plasma membrane [17], the trafficking of the fatty acid transporter FAT/CD36 [18], the regulation of insulin granule exocytosis in pancreatic β-cells [19], the translocation of the V-ATPase from an intracellular pool to the plasma membrane in response to acidosis in salivary duct epithelial cells [20] and, as we have previously demonstrated, the insulin-stimulated trafficking of the glucose transporter GLUT4 to the plasma membrane of adipocytes [21].

Rip11 itself translocates from an intracellular compartment(s) to the plasma membrane in response to insulin and phorbol esters [14,21]. In response to phorbol esters this translocation requires the presence of

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an intact Rip11 C2 domain, a region which binds phosphoinositides and phosphatidic acid in vitro [14]. Insulin-induced translocation is specific to Rip11, as it is not seen with any of the other class I FIPs [21]. However, the mechanism(s) by which these stimuli increase the translocation of Rip11 to the plasma membrane is not yet understood.

We previously reported that the effect of insulin on the translocation of a GFP–Rip11 chimera to the plasma membrane can be inhibited by wortmannin, a PI3-kinase inhibitor, and mimicked through over-expression of a constitutively-active mutant of Akt (Myr–Akt) [21]. This suggests that the phenomenon is PI3-kinase and Akt-dependent, however wortmannin is an inhibitor of all three classes of PI3-kinases [22,23] including the Class II and III isoforms that generate phosphatidylinositol 3-phosphate on intracellular vesicles. Furthermore, since our original study was published a highly selective Akt inhibitor, MK2206 [24] has become available making it easier to explore the acute regulation of the phenomenon by Akt. The trafficking mechanism deployed by Rip11 in response to insulin is also not well understood. Given the previously identified role of Rip11 in GLUT4 translocation, together with our observation that Rip11 and GLUT4 colocalise on a small proportion of intracellular vesicles, we initially reasoned that Rip11 might translocate to the plasma membrane by 'piggy-backing' on GLUT4 vesicles. Thus in this study we examined both the signalling and trafficking mechanisms involved in more detail.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were from Aventi Polar Lipids.

All other materials and reagents were from Sigma unless otherwise stated. Plasmids encoding mCherry–Rab11 and mCherry–Rab4 were kindly provided by Peter Cullen (University of Bristol, Bristol, UK).

2.2. Molecular cloning

The mutations in the Rip11 C2 domain were generated by gene synthesis (Genscript), based on the human sequence of Rip11 (amino acids 2–221; GenBank accession number AF334812). Codon optimization was undertaken essentially to decrease the %GC content and to facilitate subsequent cloning and site-directed mutagenesis. The wild-type and R52A/K54A mutant fragments were cloned in frame in the pGEX3X and peGFP-C1 vectors. To generate the full-length constructs (i.e. 2–653), the corresponding fragments 2–221 (WT or R52A/K54A) were excised from the peGFP-C1 using *Bam*HI and subcloned in frame into the C-terminal part of the Rip11 backbone (219–653) in the peGFP vector described in [14], generating a GFP–Rip11 2–653 construct. This construct was then excised from the peGFP-C1 vector and subcloned into the pmCherry-C1 vector to generate mCherry–Rip11.

2.3. Cell culture

3T3–L1 adipocytes were cultured, electroporated and treated as described before [21]. Stable cell lines were obtained by lentiviral-mediated transduction of undifferentiated 3T3–L1 by standards methods as described in [25,26]. Lentiviral plasmids were gifts of Dr. Giles Cory (University of Exeter, Exeter, UK).

2.4. Live cell imaging and immunofluorescence

For immunofluorescence, cells grown on glass coverslips were treated as indicated in figure legends, and fixed with 4% paraformaldehyde for 20 min. Coverslips were mounted in Mowiol and imaged on a Leica TCS-SP5 AOBS scanning confocal microscope.

Total internal reflection fluorescence (TIRF) microscopy of live cells was performed at 37 °C on an AM TIRF multi-colour system (Leica) attached to a DMI 6000 inverted epifluorescence microscope (Leica) using a 100× oil lens (NA = 1.46). The imaging medium consisted of phenol red-free DMEM/F12 (11,039, Invitrogen). Images were captured using an EM-CCD camera (Hamamatsu) between approx. 2–3 fps at a penetration depth of 90 nm. EGFP was excited at a wavelength of 488 nm and emission wavelengths were collected between 507 and 543 nm; mCherry was excited at a wavelength of 561 nm and emission wavelengths were collected between 584 and 616 nm. When required, cells were treated with 86 nM insulin on the microscope using a home-made perfusion system.

For inhibition of endocytosis, 3T3–L1 adipocytes stably expressing GFP–Rip11 WT were seeded on glass coverslips, and serum-starved for 3 h. The cells were then treated with vehicle (control), insulin (87 nM), Mitmab (10 or 40 μM) for 20 min at 37 °C. Alexa-Fluor™-633-transferrin (Invitrogen) was added for 20 min at 37 °C in the continuous presence of the drugs. The cells were then washed in PBS and PFA-fixed.

The amount of Rip11 at the cell surface was calculated using Volocity (PerkinElmer) by manually drawing around individual cells and calculating the fluorescence intensity from the whole cell, and then automatically contracting the region of interest by a set number of pixels in order to exclude the plasma membrane. The intensity within the inner ring was subtracted from the outer ring to give the amount of Rip11 within the plasma membrane and this value was normalised to total cellular levels of Rip11 (cell surface/total).

All images used in this manuscript were processed using Photoshop 6.0 (Adobe) and montages generated using Adobe Illustrator (Adobe). In Fig. 3 the images were subjected to contrast enhancement for illustrative purposes only, with all images being processed for display in an identical manner.

2.5. Recombinant protein purification

Recombinant GST-fusion proteins were produced and purified as described before [27]. Briefly, *Escherichia coli* BL21(DE3) (Agilent Technologies) were transformed by pGEX3X vectors containing the coding sequence for the Rip11 C2 domain (residues 2–221, WT or PI mutant) and protein expression was induced by adding 0.4 mM IPTG for 3 h at 30 °C. Bacteria were pelleted by centrifugation, washed with ice-cold phosphate buffered saline (PBS), and lysed by sonication in buffer [PBS, 0.4 mM EDTA, 2 mM DTT, 1 mM ATP, 1% Triton X100, 10 U/ml DNase I, Proteases Inhibitors Cocktail V (Calbiochem)]. The lysate was clarified by centrifugation at 10,000 g for 10 min at 4 °C, and incubated with gluthatione-Sepharose beads (GE Healthcare). The beads were washed extensively in buffer [PBS, 0.4 mM EDTA, 1% Triton X-100] and then in buffer A [50 mM Hepes pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$]. Recombinant proteins were eluted in buffer A supplemented with 10 mM reduced glutathione.

2.6. Protein–lipid overlay assay

Protein–lipid overlay assay was essentially done as described elsewhere [14]. Briefly, PIP Strips™ (Invitrogen — Molecular Probes) were blocked in buffer A plus 0.1% Tween 20 containing 3% BSA, incubated overnight at 4 °C with 1.5 μg/ml GST fusion proteins in buffer A plus 0.1% Tween 20 plus 3% BSA and washed extensively. Bound GST recombinant proteins were detected using an HRP-coupled anti-GST antibody (B-14, Santa Cruz Biotechnology Inc).

2.7. Neutral phospholipid binding assay

Neutral phospholipid binding assay was essentially done according to [16]. Briefly, PC:PE liposomes in a ratio of 80:20 were made by sonication, incubated in Buffer A + 1 mM DTT for 30 min at RT with recombinant GST or GST–Rip11 C2 domain (WT or PI mutant). After sedimentation by ultracentrifugation at 70,000 rpm for 10 min at 4 °C