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RESEARCH ARTICLE

Endomembrane PtdIns(3,4,5)P_3 activates the PI3K–Akt pathway

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ABSTRACT

PKB/Akt activation is a common step in tumour growth, proliferation and survival. Akt activation is understood to occur at the plasma membrane of cells in response to growth factor stimulation and local production of the phosphoinositide lipid phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] following phosphoinositide 3-kinase (PI3K) activation. The metabolism and turnover of phosphoinositides is complex – they act as signalling molecules as well as structural components of biological membranes. The localisation and significance of internal pools of PtdIns(3,4,5)P_3 has long been speculated upon. By using transfected and recombinant protein probes for PtdIns(3,4,5)P_3, we show that PtdIns(3,4,5)P_3 is enriched in the nuclear envelope and early endosomes. By exploiting an inducible dimerisation device to recruit Akt to these compartments, we demonstrate that Akt can be locally activated in a PtdIns(3,4,5)P_3-dependent manner and has the potential to phosphorylate compartmentally localised downstream substrates. This could be an important mechanism to regulate Akt isoform substrate specificity or influence the timing and duration of PI3K pathway signalling. Defects in phosphoinositide metabolism and localisation are known to contribute to cancer, suggesting that interactions at subcellular compartments might be worthwhile targets for therapeutic intervention.

KEY WORDS: Protein kinase B, Akt, Rapalogue dimerisation, Phosphoinositide, Subcellular compartment

INTRODUCTION

Protein kinase B (PKB)/Akt (for which there are three isoforms with differential tissue expression; Akt1, Akt2, and Akt3) is a member of the AGC kinase superfamily and is activated downstream of many growth factor and hormone receptors as a result of phosphoinositide 3-kinase (PI3K) activation (Franke et al., 1995). Over the past 20 years, intense efforts have been made to understand the roles of Akt in development, signalling, and disease. Akt phosphorylates a diverse set of substrates involved in many fundamental aspects of cell biology, including growth, survival, proliferation, angiogenesis, migration and metabolism (Manning and Cantley, 2007).

The mechanism of Akt activation has been reviewed comprehensively elsewhere (Calleja et al., 2009b; Hanada et al., 2004). Akt is recruited to phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] and phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)P_2] through an interaction with its pleckstrin homology (PH) domain, resulting in a conformation change that permits phosphorylation of the kinase domain at two regulatory sites, T308 by phosphoinositide-dependent kinase 1 (PDK1, also known as PDPK1) (Calleja et al., 2007; Milburn et al., 2003) and S473 by mammalian target of rapamycin complex 2 (mTORC2) (Sarbassov et al., 2005). Phosphorylation at both sites is required for full kinase activity.

The most prominent site of Akt recruitment and activation is the plasma membrane; however, this might not be the only site. Phosphoinositides are readily interconverted through the actions of lipid kinases and lipid phosphatases in endomembrane compartments (Lindsay et al., 2006; Watt et al., 2004), and their presence serves to determine the physical properties of the membranes in which they reside (Dumas et al., 2010; Larijani and Dufourc, 2006; Larijani and Poccia, 2012; Zhendre et al., 2011). In addition, their roles in signalling suggest that Akt might be activated on internal membranes. Compartmentally localised Akt activation could be important for spatial or temporal regulation, for substrate specificity, or for isoform-specific responses to particular stimuli. Defects in phosphoinositide metabolism have been reported in a number of human diseases (Pendaries et al., 2003) and aberrant Akt activation is a key step in tumour development. Therefore, there might be a link between defects in phosphoinositide metabolism and pathological misregulation or mislocalisation of PI3K pathway activation.

Of the possible endomembranes involved in intracellular signalling, the endosomes and nuclear envelope stand out as likely candidates, owing to their links with internalised activated growth factor receptor complexes, such as the epidermal growth factor (EGF) receptor (Levkowitz et al., 1999). Endocytosed receptor complexes remain capable of signalling, resulting in phosphorylation of receptors, signalling effectors and downstream substrates at internal sites (Er et al., 2013; Jiang and Sorkin, 2002; Kermorgant et al., 2004; Lampugnani et al., 2006).

The nuclear envelope also contains significant amounts of phosphoinositides and their derivatives (Byrne et al., 2007; Domart et al., 2012; Irvine, 2006; Larijani et al., 2000). Given that the nucleus contains several substrates of Akt, including proteins involved in mRNA processing and DNA damage repair (Blaustein et al., 2005; Boronenkov et al., 1998; Bozulic and Hemmings, 2009; Okada and Ye, 2009), nuclear-localised Akt activation remains an intriguing possibility. Several studies have shown that activated Akt exists in endosomes (Schenck et al., 2008; Zoncu et al., 2009). However, internal activation of Akt (as opposed to translocation of activated Akt from the plasma membrane) has never been demonstrated.

In this study, we investigated the potential for intracellular Akt activation in response to growth factor stimulation using a dimerisation tool to inducibly and acutely recruit Akt to the endosomes or to the nuclear envelope. We quantified Akt regulatory site phosphorylation under a variety of conditions to demonstrate that Akt can be locally activated on endomembranes in response to

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PtdIns(3,4,5)P_3 binding, that endosomal Akt colocalises with its upstream activator PDK1, and that endosomal Akt can phosphorylate substrates *in situ*.

RESULTS

RFP–FRB–Akt can be inducibly and acutely recruited to intracellular membranes

In order to determine whether Akt could be activated on endomembranes, we used a chemically inducible dimerisation system to recruit Akt1 to early endosomes or to the nuclear envelope (Fig. 1A,B), consisting of a membrane anchor containing FKBP, which targeted a compartment of interest, and a cytoplasmically expressed partner, containing FRB fused to human Akt1.

Rab5 and the lamin B receptor (LBR) were used to target the membrane anchor to the early endosomes and the nuclear envelope, respectively. Addition of rapalogue induced translocation of RFP–FRB–Akt from the cytoplasm to either the early endosomes or the nuclear envelope (Fig. 1C).

Pools of PtdIns(3,4,5)P_3 exist in the early endosomes and nuclear envelope

Given that Akt activation has been shown to be PtdIns(3,4,5)P_3 dependent, we investigated whether endomembrane pools of PtdIns (3,4,5)P_3 could be detected in our cells, using the PH domain of GRP1 (also known as CYTH3) as a probe. This probe was selected for its high specificity but has a relatively low affinity for PtdIns

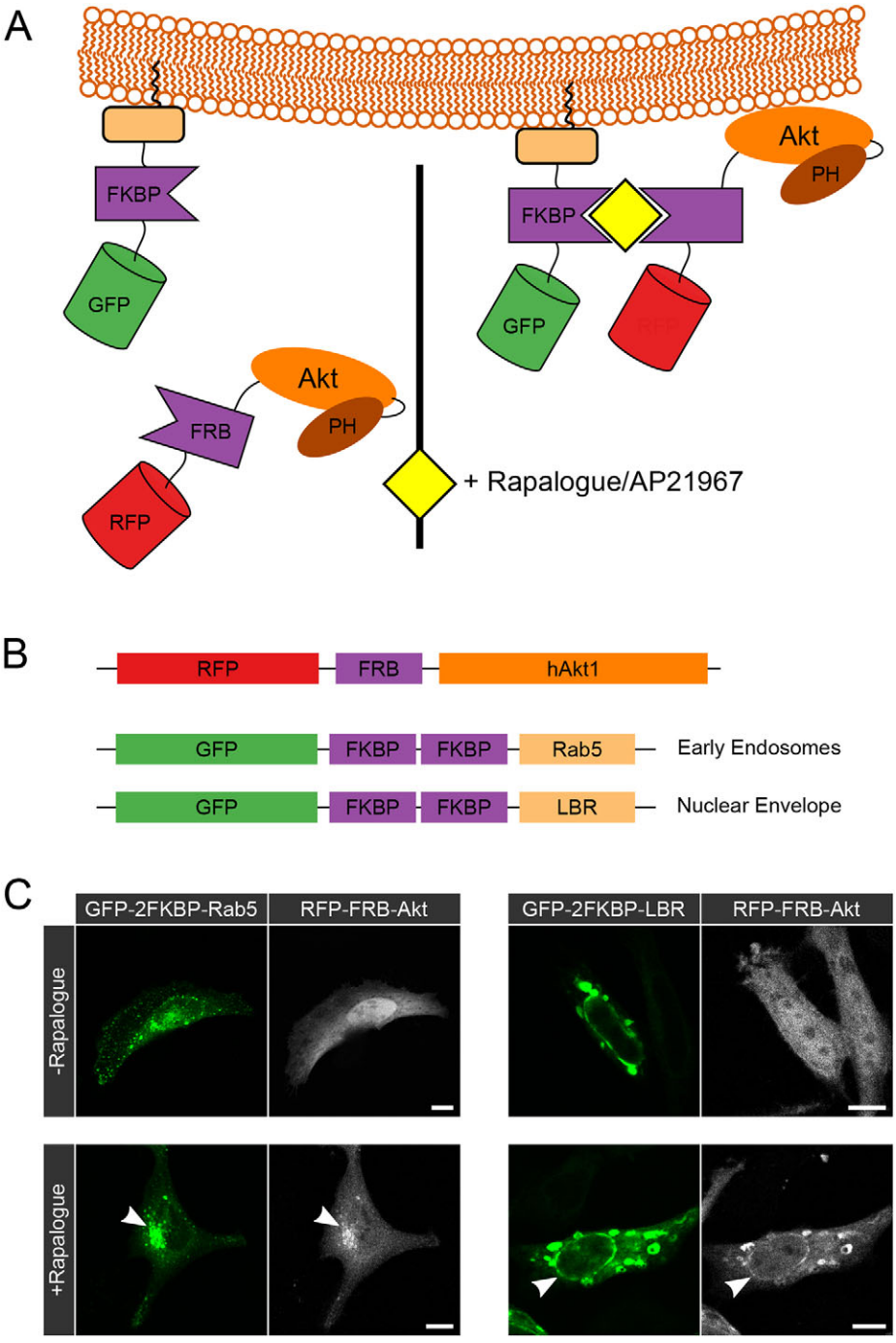

Fig. 1. Recruitment of RFP–FRB–Akt to the early endosomes or nuclear envelope. (A,B) Schematic diagram of the dimerisation fusion constructs based on Spencer et al. (1993). One component, FRB, is expressed in the cytoplasm attached to the protein of interest Akt (human Akt1, hAkt); the other component, tandem FKBP, is anchored to an intracellular membrane – either through Rab5 to target the early endosomes or LBR to target the nuclear envelope. Upon rapalogue addition, the FRB and FKBP domains interact with high affinity, recruiting Akt to the appropriate membrane compartment. The dimerisation constructs are fused to GFP or RFP for visualisation. (C) Treatment of cells with rapalogue (500 nM, 90 min) induced translocation of RFP–FRB–Akt from the cytoplasm to the early endosomes or to the nuclear envelope (arrows). Scale bars: 10 µm.

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(3,4,5)P_3 (50–100 nM), as opposed to the PH domain of PDK1 (0.2–4 nM); therefore, unlike high-affinity PH domains, it will not sequester PtdIns(3,4,5)P_3 to an exclusive compartment such as the plasma membrane.

We have previously observed differences in the localisation of transfected probes and probes applied as recombinant proteins – for example, the transfected PtdIns(4,5)P_2 probe PLCδ1^{PH} labelled only the plasma membrane, while purified recombinant PLCδ1^{PH} revealed PtdIns(4,5)P_2 at the plasma membrane, nuclear envelope, and other internal membranes (Domart et al., 2012). We therefore compared the localisation of transfected GFP–GRP1^{PH}, and the recombinant GST–GRP1^{PH} probe purified from *E. coli* (Fig. 2).

When transfected into live cells, the GFP–GRP1^{PH} probe exhibited a PtdIns(3,4,5)P_3-sequestering effect at the plasma membrane, which was not observed with the PtdIns(3,4,5)P_3 non-binding K273A mutant (Fig. 2A). Both the mutant and wild-type transfected probes accumulated non-specifically in the nucleus. Meanwhile, the recombinant GST–GRP1^{PH} probe applied to fixed cells labelled the nuclear envelope and the endoplasmic reticulum. Incubation of the recombinant probe in the presence of an excess of the PtdIns(3,4,5)P_3 headgroup InsP(1,3,4,5)P_4 resulted in the loss of structure-specific binding (Fig. 2B). Colocalisation with the Mab414 antibody (raised against nuclear pore complex components), was used to confirm localisation of the recombinant probe to the nuclear envelope (Fig. 2C). The specificity of the recombinant probe for PtdIns(3,4,5)P_3 was validated *in vitro* using a phosphoinositide strip (supplementary material Fig. S1). Thus, there appear to be substantial pools of intracellular PtdIns(3,4,5)P_3 in the absence of growth factor stimulation. These pools are likely to be regulated separately from the plasma membrane pool, and their presence suggests that Akt could be selectively recruited and activated at the early endosomes or nuclear envelope, which are currently not regarded as sites of Akt activation.

Akt can be phosphorylated on endomembrane compartments

Given that PtdIns(3,4,5)P_3 could be detected in endomembranes, we next investigated whether Akt could be locally activated in these compartments. Using the inducible dimerisation system to recruit Akt to either the early endosomes or the nuclear envelope, we quantified Akt activation by monitoring the phosphorylation status at the T308 (activation loop, pT308) and S473 (hydrophobic motif, pS473) regulatory sites (Fig. 3).

The phosphorylation status of Akt recruited to the early endosomes was measured by calculating the intensity ratio of antibody-labelled pT308 or pS473 compared to that of RFP–FRB–Akt in individual endosomes (as identified by the presence of GFP–2FKBP–Rab5). This method allowed us to determine the phosphorylation status of Akt in tightly defined regions of the cell, which would not have been possible by biochemical cell fractionation.

Representative images show that Akt recruited to the early endosomes was phosphorylated (Fig. 3A), and the accompanying graphs indicate the phosphorylation status of Akt under different stimulation conditions – unstimulated, after EGF stimulation, and after EGF stimulation in the presence of the PI3K inhibitor LY294002. Following stimulation, Akt recruited to the early endosomes was phosphorylated on S473; LY294002 pre-treatment reduced phosphorylation on both sites, suggesting that Akt activation was dependent on the presence of PtdIns(3,4,5)P_3 in this compartment. The lack of phosphorylation of the Akt mutants T308A and S374A demonstrated the specificity of the antibodies and validated the use of this method to quantify Akt phosphorylation status.

Similar experiments were performed after recruiting Akt to the nuclear envelope (Fig. 3B). In this case, the average intensity around each nuclear envelope (as defined by the presence of GFP–2FKBP–LBR) was used to calculate the Akt phosphorylation status. Phosphorylation at T308 and S473 increased upon EGF stimulation and decreased in response to LY294002 pre-treatment, suggesting that nuclear-envelope-localised PtdIns(3,4,5)P_3 is required for Akt activation.

Our experiments showed that RFP–FRB–Akt acutely recruited to either the early endosomes or to the nuclear envelope was phosphorylated in a PI3K-dependent manner. These data, together with the localisation of PtdIns(3,4,5)P_3 in these membranes (Fig. 2),

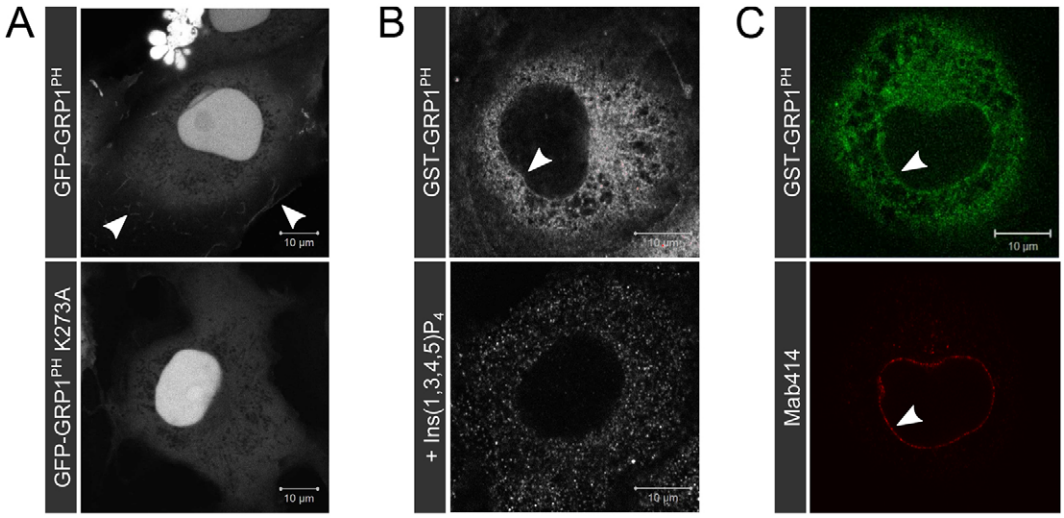

Fig. 2. PtdIns(3,4,5)P_3 localises to subcellular compartments. (A) Cos7 cells were transiently transfected with GFP–GRP1^{PH} or the PtdIns(3,4,5)P_3 non-binding mutant K273A. Arrowheads highlight the plasma membrane. (B) Cos7 cells were fixed and probed with the GST–GRP1^{PH} recombinant probe (upper panel) or the recombinant probe with a 10-fold molar excess of Ins(1,3,4,5)P_4. The arrowhead indicates the nuclear envelope. (C) Cos7 cells were fixed and probed wtith the GST–GRP1^{PH} recombinant probe and colabelled with a nuclear pore complex antibody (Mab414). Arrowheads indicate the nuclear envelope.

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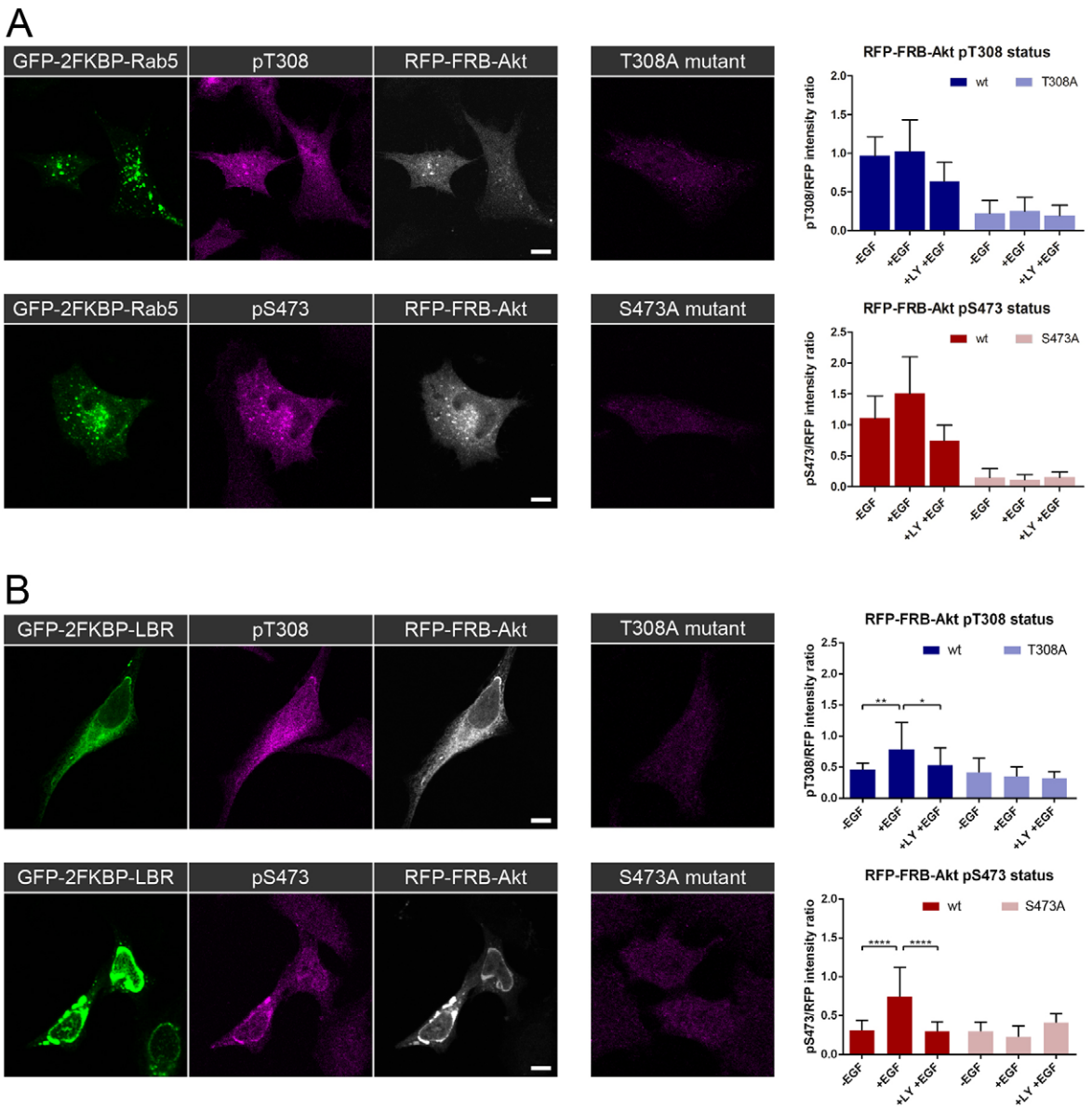

Fig. 3. Phosphorylation status of Akt recruited to the early endosomes and nuclear envelope. (A) HeLa cells transiently coexpressing the dimerisation constructs GFP–2FKBP–Rab5 and RFP–FRB–Akt (or the non-phosphorylatable T308A or S473A mutants) were stimulated with EGF (100 ng/ml, 2 min) or pre-treated with LY294002 (50 µM, 20 min) in the presence of rapalogue (500 nM, 90 min). Cells were fixed and labelled with anti-pT308 (upper panel) or anti-pS473 (lower panel) antibodies. Images show cells stimulated with EGF; the non-phosphorylatable mutant is shown to the right. Graphs show the mean±s.d. relative phosphorylation of vesicles (calculated as described in Materials and Methods) for each condition (n=10–20 cells, 50–200 vesicles per cell). (B) HeLa cells transiently coexpressing GFP–2FKBP–LBR and RFP–FRB–Akt were treated as in A. Graphs show the mean±s.d. relative phosphorylation around a profile of the nuclear envelope (n=10–20 cells). *P<0.05; **P<0.01; ****P<0.0001 (Student's t-test). LY, LY294002; wt, wild-type Akt.

suggest that the mechanism of action of Akt activation was through binding of the PH domain to PtdIns(3,4,5)P_3, similar to events occurring at the plasma membrane. Subsequent experiments focussed on the early endosome compartment.

Endosomal Akt activation is dependent on class I PI3K activity

The sensitivity of both nuclear-envelope- and endosome-localised Akt to LY294002 pre-treatment suggested a dependence on PI3K-mediated PtdIns(3,4,5)P_3 production. However, LY294002 is non-selective with respect to the different PI3K isoforms and also has been shown to inhibit several other kinases, including glycogen synthase kinase 3 (GSK3) and mTOR, which are involved in the regulation of cellular metabolism and proliferation (Gharbi et al., 2007). In order to narrow down the requirement to class I PI3K, which acts directly downstream of growth factor receptor stimulation (Hawkins et al., 1992), we pre-incubated cells with PIK-75, a highly selective inhibitor of the p110α subunit of PI3K (IC_{50} 6 nM; Hayakawa et al., 2007), which does not inhibit the p110β, p110γ or p110δ subunits, or class II or III PI3Ks (Knight et al., 2006).

PIK-75 pre-treatment reduced the phosphorylation of endosome-recruited Akt at both T308 and S473 (Fig. 4A), confirming that phosphorylation was dependent on class I PI3K and suggesting that PtdIns(3,4,5)P_3 binding was necessary for endosomal Akt activation.

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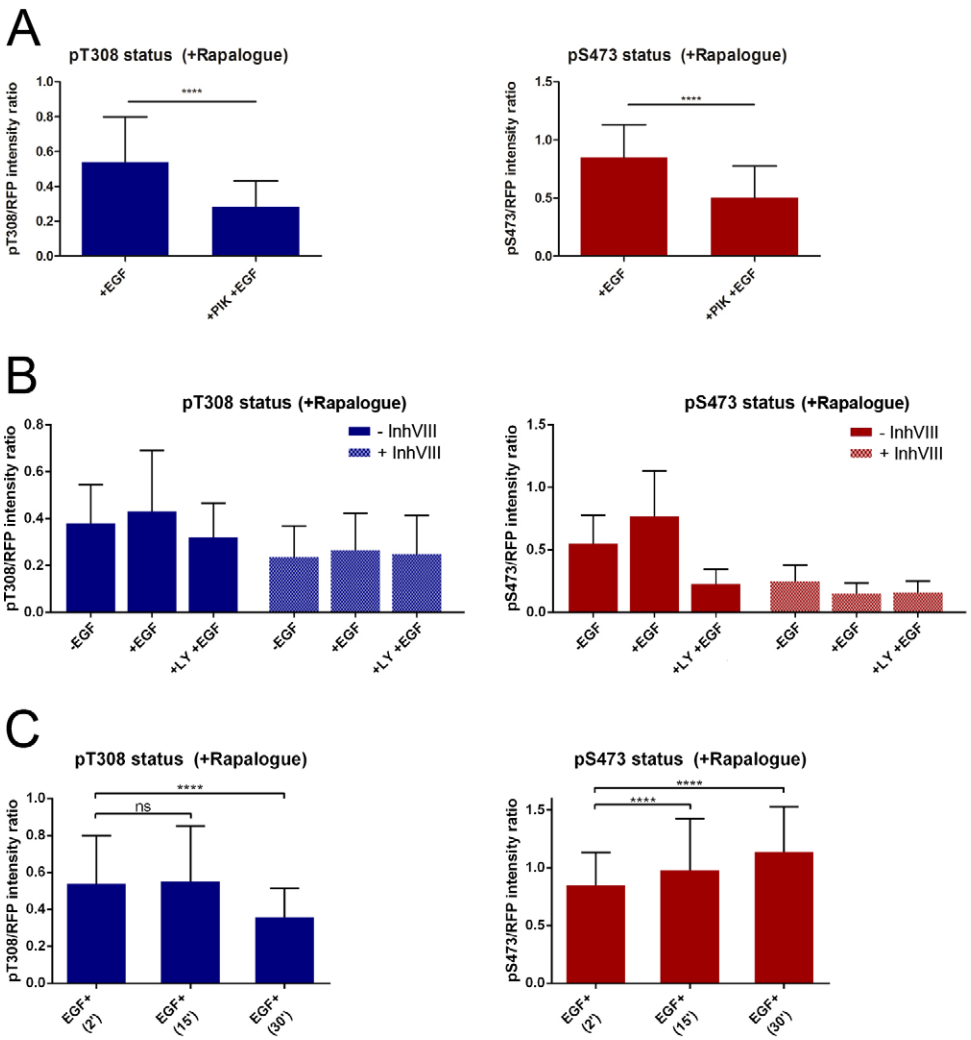

Fig. 4. PtdIns(3,4,5)P_3 dependence of endosomal Akt activation. (A) HeLa cells coexpressing RFP–FRB–Akt and GFP–2FKBP–Rab5 were stimulated with EGF (100 ng/ml, 2 min) or pre-treated with PIK-75 (500 nM, 20 min) before EGF stimulation, in the presence of rapalogue (500 nM, 90 min). The phosphorylation status of endosome-recruited RFP–FRB–Akt was quantified as described in the Materials and Methods. (B) HeLa cells coexpressing RFP–FRB–Akt and GFP–2FKBP–Rab5 were stimulated with EGF (100 ng/ml, 2 min) or pre-treated with LY294002 (50 µM, 20 min) before EGF stimulation, in the presence of rapalogue (500 nM, 90 min); conditions were set up in the presence and absence of the Akt-specific Inhibitor VIII (5 µM, 30 min). The phosphorylation status of endosome-recruited RFP–FRB–Akt was quantified as described in the Materials and Methods. (C) HeLa cells coexpressing RFP–FRB–Akt and GFP–2FKBP–Rab5 were treated with rapalogue (500 nM, 90 min) before stimulation with EGF (100 ng/ml) for 2, 15, or 30 min. The phosphorylation status of endosome-recruited RFP–FRB–Akt was quantified as described in the Materials and Methods. Graphs show mean±s.d. (n=10–20 cells, 50–200 vesicles per cell). ****P<0.0001; ns, not significant (Student's t-test). InhVIII, Akt Inhibitor VIII; LY, LY294002; ns, not significant; PIK, PIK-75.

PtdIns(3,4,5)P_3 binding is necessary for endosomal Akt activation

At the plasma membrane, Akt is known to undergo a PtdIns(3,4,5)P_3-mediated change in conformation; we next investigated whether endosome-localised Akt undergoes the same change. The PH domain of Akt serves two functions prior to stimulation: intramolecular interactions between the PH and kinase domains shield the ATP-binding site and block the T-loop from PDK1 access, locking the kinase in an inactive (PH-in) conformation and preventing premature activation (Calleja et al., 2007, 2009a; Wu et al., 2010). Upon growth factor stimulation and PI3K activation, binding of the PH domain to PtdIns(3,4,5)P_3 triggers both plasma membrane translocation and a conversion to a PH-out conformation, permitting T-loop phosphorylation by co-recruited PDK1 (Calleja et al., 2009a; Currie et al., 1999; Landgraf et al., 2008; Milburn et al., 2003).

In our system, rapalogue addition induces RFP–FRB–Akt endomembrane translocation, but does not satisfy the requirement for PtdIns(3,4,5)P_3 binding. To explore whether functional PtdIns(3,4,5)P_3 binding at the early endosomes was required for localised Akt activation, or whether Akt activation was solely due to its forced recruitment to this compartment, we used the specific Akt1 and Akt2 allosteric inhibitor Akt Inhibitor VIII. In contrast to classical ATP competitive inhibitors, Akt inhibitor VIII works by locking Akt in the inactive, PH-in conformation (Calleja et al., 2009a; Lindsley et al., 2005; Wu et al., 2010). We postulated that endosome-recruited Akt capable of interacting normally with PtdIns(3,4,5)P_3 (and therefore undergoing a PH-in to PH-out conformation change) would remain sensitive to inhibition by Akt Inhibitor VIII.

The phosphorylation status of Akt S473 was monitored in the early endosomes after EGF stimulation, in the absence or presence of Akt Inhibitor VIII (Fig. 4B). As previously shown, phosphorylation at S473 was sensitive to EGF stimulation and PI3K inhibition by LY294002. In the presence of Akt inhibitor VIII, there was a decrease in S473 phosphorylation, which was no longer affected by either growth factor stimulation or PI3K inhibition. (This effect was less pronounced for T308 phosphorylation.) These results indicate that the inhibitor had locked Akt in the inactive PH-in conformation, rendering it functionally unresponsive to the presence of PtdIns(3,4,5)P_3. It is important to note that the combination of rapalogue-induced stable recruitment of RFP–FRB–Akt to the endosome compartment and quantitative image analysis ensured that the assay reported on *in situ* Akt activation status.

The source of PtdIns(3,4,5)P_3 on early endosomes is class I PI3K

The most likely source of PtdIns(3,4,5)P_3 in the early endosomes is the canonical PI3K pathway. Previous studies using PtdIns(3,4,5)P_3 sensors as spatiotemporal probes have been informative as to the organisation of the PI3K pathway in subcellular localisations (Antal

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and Newton, 2013), showing that after growth factor stimulation, PtdIns(3,4,5)P_3 levels peaked strongly and returned to basal levels within minutes. However, although receptor endocytosis occurs on a longer timescale, measurement of Akt phosphorylation in endocytosed complexes has previously not been possible, preventing the examination of endomembrane-localised Akt phosphorylation kinetics. In this study, we quantified only the endosome-recruited pool of Akt, disregarding the remaining population.

After sustained EGF stimulation, changes in endosome-recruited Akt phosphorylation were maintained for up to 30 min (Fig. 4C). Phosphorylation at T308 was maintained for at least 15 min before decreasing, whereas S473 phosphorylation gradually increased over the 30-min timecourse.

These results suggest that Akt activated at the endosomal compartment is regulated separately from the pool at the plasma membrane, and that T308 and S473 are regulated with different kinetics. Earlier experiments showed that LY294002 or PIK-75 treatment blocked endosome-localised Akt phosphorylation, indicating that maintenance of Akt phosphorylation was more likely to be due to the prolonged presence of PtdIns(3,4,5)P_3 than to a lack of dephosphorylation of Akt by phosphatases.

RFP–FRB–Akt colocalises with its upstream activator, PDK1

Akt T308 phosphorylation is mediated by its upstream activator PDK1 (Alessi et al., 1997; Stephens et al., 1998). We therefore investigated whether PDK1 might phosphorylate Akt directly on the early endosomes.

We co-transfected Myc–PDK1 into cells expressing the inducible dimerisation system in order to observe whether PDK1 would colocalise with RFP–FRB–Akt in the early endosomes (Fig. 5A). Upon EGF stimulation, Myc–PDK1 translocated to the plasma membrane as well as to intracellular vesicles containing RFP–FRB–Akt. Given that the 90-min incubation with rapalogue prevents exchange of the recruited RFP–FRB–Akt with the cytoplasm or plasma membrane, this experiment suggests that endosomal RFP–FRB–Akt can be phosphorylated *in situ* by colocalised PDK1.

Endosomal Akt is able to phosphorylate a substrate peptide

Finally, we demonstrated that endosome-localised RFP–FRB–Akt was enzymatically active and capable of phosphorylating a potential substrate. The traditional biochemical kinase assay measuring incorporation of ^{32}P-ATP into the Crosstide substrate peptide (derived from the Akt phosphorylation site on GSK3β) was not feasible here; instead, we incorporated the Crosstide peptide sequence into the endosome-anchored dimerisation construct (Fig. 5B). Phosphorylation of the Crosstide sequence was detected and quantified by immunofluorescence in the region of recruited vesicles, in a similar manner to the Akt phosphorylation status (Fig. 5C). There was a significant increase in the phosphorylation status of the Crosstide sequence upon recruitment of RFP–FRB–Akt to the early endosomes, however the level of phosphorylation was not responsive to EGF stimulation – likely due to the enforced proximity of enzyme and substrate on the endosomes, resulting in constitutive substrate phosphorylation.

DISCUSSION

Using an inducible dimerisation tool to restrict the localisation of Akt to either the early endosomes or the nuclear envelope, we

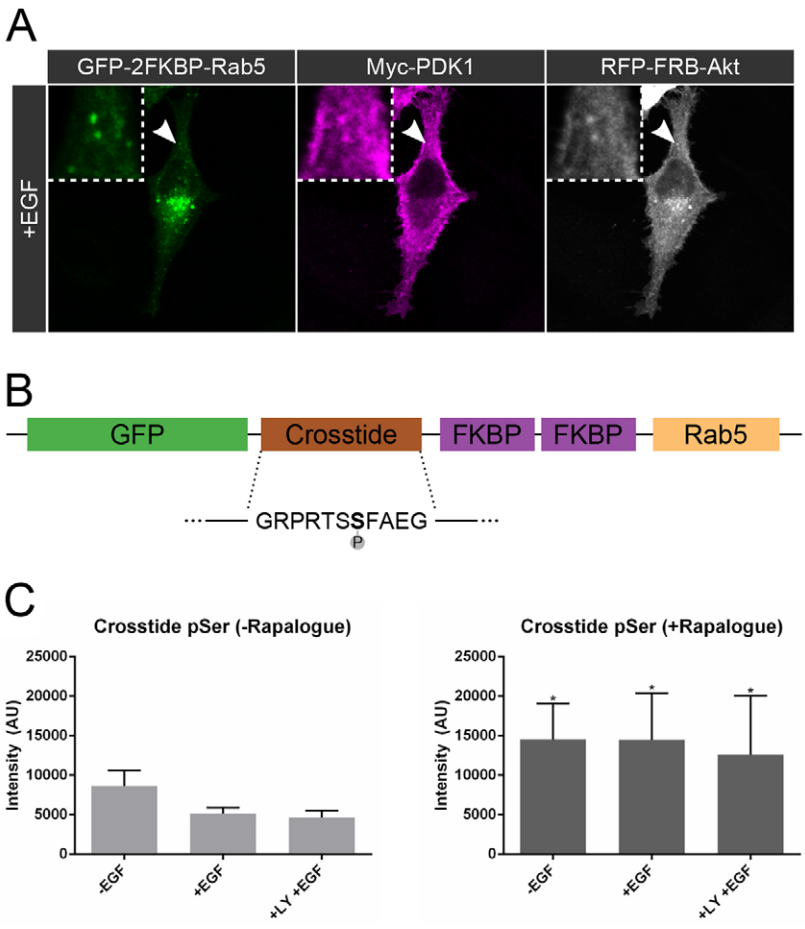

Fig. 5. Endosome-recruited Akt colocalises with PDK1 and phosphorylates a substrate peptide. (A) HeLa cells were transfected with RFP–FRB–Akt (white), GFP–2FKBP–Rab5 (green) and Myc–PDK1 (magenta). Cells were stimulated with EGF (100 ng/ml, 2 min) in the presence of rapalogue (500 nM, 90 min). Arrowheads mark the expanded region shown in the inset. (B) Schematic of the GFP–Crosstide–2FKBP–Rab5 recruitment construct showing Akt substrate peptide sequence (Crosstide) corresponding to residues of GSK3β surrounding the Ser9 phosphorylation site. (C) HeLa cells coexpressing RFP–FRB–Akt and GFP–Crosstide–2FKBP–Rab5 were stimulated with EGF (100 ng/ml, 2 min) in the presence of rapalogue (500 nM, 90 min). Cells were labelled with an anti-GSK-3β pSer9 antibody, and the mean±s.d. intensity of pSer9 staining on RFP–FRB–Akt-positive vesicles was measured (n=10–20 cells, 50–200 vesicles per cell). *P<0.05 (compared to equivalent condition in the absence of rapalogue, Student's t-test).

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demonstrated that Akt can potentially be activated by internal pools of PtdIns(3,4,5)P_3. Although the RFP–FRB–Akt dimerisation construct was ectopically expressed and induced to interact with subcellular compartments, this tool allowed us to investigate localised processes in cells without resorting to biochemical fractionation of these compartments, and thus provided a valuable insight into the potential for intracellular Akt activation on intact subcellular compartments.

We showed that Akt activation was dependent on the presence of PtdIns(3,4,5)P_3 generated by class I PI3K, and colocalised PDK1 might be responsible for phosphorylating the Akt T-loop. Localised Akt activity was inferred from the increased T308 and S473 phosphorylation status, and by downstream phosphorylation of the endosome-anchored Crosstide peptide. We speculate that intracellular Akt activation might contribute to the substrate specificity of the different isoforms, or to the temporal regulation of PI3K signalling; alternatively, it might play a pathogenic role in certain diseases in which normal phosphoinositide metabolism is misregulated.

In this study, we focussed on the early endosome compartment, which has well-defined links to the PI3K pathway. In particular, a number of recent studies have suggested that the interconversion of phosphoinositides regulates both endosome maturation and Akt substrate specificity. In zebrafish, knockdown of APPL1, an effector of Rab5 found in early endosomes, results in increased apoptosis due to a reduction in Akt signalling; however, tissue growth and cell proliferation, which are also regulated by Akt, are unaffected. This effect was attributed to differential Akt substrate specificity with regard to GSK3β, which localised with APPL1 at the early endosomes, and TSC2, which did not (Schenck et al., 2008).

Phosphoinositides also play a central role in regulating trafficking. Intracellular compartments can be identified by the presence of particular phosphoinositide species – for example, PtdIns(3)*P* and PtdIns(3,5)P_2 in the early and late endosomes, respectively – whose role is to recruit lipid-binding proteins with specific functions (Di Paolo and De Camilli, 2006). The fate of early endosomes is strongly influenced by the presence of PtdIns(3)*P*; the loss of APPL1, coinciding with phosphorylation of PtdIns(3)*P* to PtdIns(3,5)P_2, appears to be the trigger for endosome maturation (Zoncu et al., 2009). Akt activity activates the PtdIns 5-kinase PIKfyve, resulting in phosphorylation of PtdIns(3)*P* to PtdIns(3,5)P_2, and is required for endosome maturation (Er et al., 2013). PTEN has also been shown to localise to early endosomes through a specific interaction with PtdIns(3)*P* (Naguib et al., 2015).

The presence of subcellular pools of PtdIns(3,4,5)P_3 therefore has implications for localised PI3K pathway signalling, as well as other phosphoinositide-regulated processes. Our study provides evidence to support a negative feedback model in which endosome-localised Akt phosphorylates specific substrates, while downregulating signalling by triggering phosphoinositide turnover, endosome maturation and receptor degradation (Fig. 6).

In the nuclear envelope, we have previously shown that phosphoinositides (and their derivative, diacylglycerol) play important structural, functional and morphological roles (Domart et al., 2012). The possibility of localised Akt activation in the nuclear envelope is therefore of great interest – although nuclear substrates of Akt have been previously reported, we are the first to demonstrate that Akt can be activated directly on the nuclear envelope in a PtdIns(3,4,5)P_3-dependent manner. This supports the idea that nuclear phosphoinositides are organised within membrane bilayers, perhaps within invaginations of the nuclear envelope protruding deep into the nuclear matrix (Malhas et al., 2011). Our results suggest there are intriguing links between receptor activation at the cell surface, phosphoinositide metabolism in intracellular compartments and nuclear functions of Akt (Bozulic and Hemmings, 2009; Okada and Ye, 2009). The route of signal transduction remains unclear, however, and might depend in part on the ability of soluble adaptor proteins to diffuse to internal sites and locally upregulate PtdIns(3,4,5)P_3 production (Wang et al., 2014).

To summarise, the requirement of PtdIns(3,4,5)P_3 binding for Akt activation is now well understood, and it is generally assumed that the plasma membrane is the only substantial site of Akt activation. Using a chemically inducible tool to acutely recruit Akt to the early endosome or nuclear envelope compartments, we have shown that Akt can be activated locally at these sites, and that activation is dependent on localised PtdIns(3,4,5)P_3 binding and

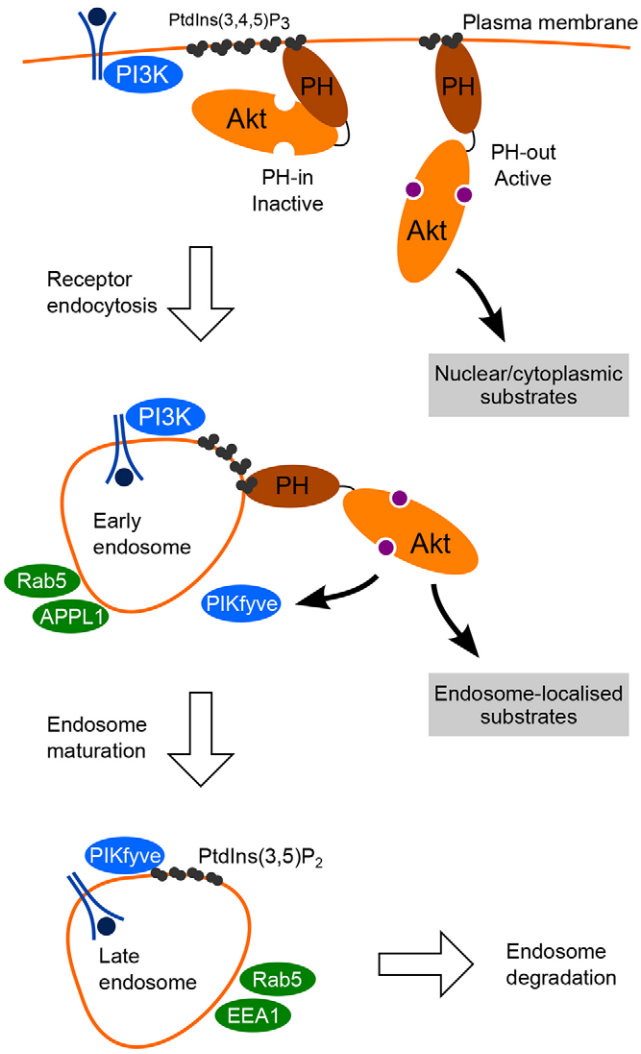

Fig. 6. Model of Akt activation at the early endosomes. The accepted model of Akt activation occurs at the plasma membrane, where PtdIns(3,4,5)P_3 binding induces a conformation change from PH-in (closed, inactive) to PH-out (open, active), which can be phosphorylated on both regulatory sites by PDK1 and mTORC2. Our experiments show that Akt activation can also occur on the early endosomes in response to cell stimulation and local production of PtdIns(3,4,5)P_3, potentially resulting in phosphorylation of endosome-localised substrates. One effect of endosome-localised Akt activation might be to regulate PIKfyve activity – phosphorylation of PtdIns(3)*P* to PtdIns(3,5)P_2 would trigger endosome maturation and degradation, thereby switching off further PI3K pathway signalling from this compartment.

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conformation change. Internal Akt activation by internalised signalling complexes or on the nuclear envelope might extend the duration of signalling induced by growth factors, or produce a qualitatively different response through the recruitment of specific substrates or activation of specific Akt isoforms. The identification of localised Akt substrates is likely to be of considerable interest, especially in light of studies demonstrating the role of isoform-specific Akt signalling in controlling proliferation and migration of tumour cell lines in three-dimensional culture (Chin and Toker, 2009, 2010).

MATERIALS AND METHODS

Tissue culture

HeLa cells were obtained from Cancer Research UK Cell Services at Clare Hall. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (Gibco, Invitrogen) supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were transfected using Invitrogen Lipofectamine LTX with Plus Reagent prepared according to manufacturer's instructions.

Inducible dimerisation system

The inducible dimerisation system contains two components: FK506-binding protein (FKBP) and a fragment of mTOR (FRB), which dimerise upon binding with FK506 (rapamycin) or the non-immunosuppressive derivative, AP21967 (rapalogue) (Spencer et al., 1993). The following constructs were prepared: RFP–FRB–Akt1, GFP–2FKBP–Rab5 (to target the early endosomes) (Fili et al., 2006), and GFP–2FKBP–LBR (lamin B receptor, to target the nuclear envelope) (Domart et al., 2012). Tandem FKBP domains were used to improve the dimerisation efficiency (Fili et al., 2006).

Dimerisation of transfected constructs was induced by addition of rapalogue (500 nM final concentration; Clontech Laboratories, Inc.) for 90 min at 37°C. The extent of dimerisation could be monitored by confocal microscopy using the GFP and RFP tags fused to each construct.

Cell stimulation and inhibitor treatment

Growth factors and/or inhibitors were added at appropriate time points during the 90-min rapalogue incubation to ensure that all conditions experienced the same total exposure to rapalogue, EGF and inhibitor. Inhibitors used were LY294002 (50 µM, 20 min incubation; Calbiochem), PIK-75 (500 nM, 20 min incubation; Cambridge Bioscience), and Akt Inhibitor VIII (5 µM, 30 min incubation; Calbiochem). Human EGF (100 ng/µl; Calbiochem) was added to the culture medium for the final 2 min before fixing, labelling and mounting cells as described below.

Recombinant GST–GRP1^{PH} purification and imaging

The GRP1^{PH} domain was used as a probe for PtdIns(3,4,5)P_3 (Gray et al., 1999; Lindsay et al., 2006). GFP–GRP1^{PH} was used for confocal imaging, and GST–GRP1^{PH} was used as a recombinant protein probe.

GST–GRP1^{PH} was expressed in chemically competent *E. coli* (DH5α, Invitrogen). Bacteria were transformed by heat shock and grown on Luria broth (LB) agar supplemented with ampicillin (100 µg/ml). A single colony was used to inoculate a 2 litre culture of LB-ampicillin and incubated at 37°C until the culture reached an optical density at 600 nm of 0.6. Protein expression was induced by adding isopropyl thiogalactopyranoside (1 mM) for 14 h at 18°C. Bacteria were centrifuged at 2000 ***g***, resuspended in 50 ml distilled H_2O, treated with lysozyme (10 mg) on ice and frozen at −20°C. Thawed bacteria were mixed with lysis buffer and subjected to probe sonication. The supernatant was loaded onto 750 µl glutathione-tagged Sepharose beads (GE Healthcare). GST–GRP1^{PH} was eluted in glutathione elution buffer on ice for 10 min. The concentration and identity of eluted protein were confirmed by the Bradford assay and western blotting (supplementary material Fig. S1). Protein mass spectrometry [nanoACQUITY UPCL (Waters Corporation) coupled to an LTQ-Orbitap XL (Thermo Fisher Scientific)] was also used to identify the purified protein. Peptides were eluted over a 30-min gradient [5–40% (vol/vol) acetonitrile]. Peak lists were extracted using Mascot distiller and searched with Mascot v.2.4.1 (Matrix Science) against the Uniprot reference database. Oxidation of methionine, was entered as a variable modification and the search tolerances were 5 ppm and 0.8 Da for peptides and fragments, respectively. Searches were compiled in Scaffold 4.1.1.

For imaging, cells were fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 min at room temperature. Cells were washed three times in Tris-buffered saline (TBS) and permeabilised in 0.5% saponin, 3% (w/v) bovine serum albumin (BSA) in TBS at room temperature for 30 min. Cells were incubated with GST–GRP1^{PH} (70 µg/ml) and antibodies raised against nuclear pore complex proteins (1:500, Mab414; Covance) diluted in permeabilisation buffer for 1 h at room temperature. Cells were washed once in TBS before incubation for 1 h at room temperature with secondary anti-GST antibody conjugated to DY488 (1:250, Abcam) and anti-mouse-IgG conjugated to Alexa Fluor 592 (1:500, Jackson Laboratories) antibodies, diluted in permeabilisation buffer. After washing in TBS, samples were mounted in 2.5% (w/v) 1,4-diazobicyclo(2.2.2)octane (DABCO) in TBS.

Phosphorylated Akt immunofluorescence

Cells were transfected and stimulated as described above, before fixation in 4% PFA in PBS (w/v) for 10 min at room temperature. Cells were washed twice in PBS, then permeabilised in freshly prepared 0.2% saponin (w/v) for 15 min, before blocking in 1% fatty-acid free BSA (w/v) for 30 min at room temperature.

Primary antibodies were diluted in 1% BSA blocking solution and incubated overnight at 4°C. Antibodies used were against Akt pS473 (D9E, Cell Signalling; 1:50 dilution), Akt pT308 (C31E5E, Cell Signalling; 1:100 dilution), and GSK-3β pSer9 (D85E12, Cell Signalling; 1:100 dilution). Secondary antibodies were diluted 1:250 in 1% BSA in PBS; samples were incubated for 1 h at room temperature. Antibodies used were donkey anti-rabbit-IgG conjugated to Alexa Fluor 647 and goat anti-mouse-IgG conjugated to Dylight405 (Jackson Laboratories). Samples were mounted in DABCO before imaging.

Imaging and quantification

Images were acquired on a Zeiss LSM Invert 710 microscope equipped with a Zeiss Plan-Apochromat 63× oil immersion objective lens (1.4 NA).

Images were analysed in ImageJ (NIH). Early endosomes were identified by the presence of GFP–2FKBP–Rab5. The intensity at the centre of each endosome in the red (RFP–FRB–Akt, labelled with RFP) and far-red (pAkt, labelled with the Alexa-Fluor-647-conjugated secondary antibody) channels was measured. An intensity ratio was obtained by dividing the pAkt intensity by the Akt intensity, giving a quantitative measure of Akt phosphorylation status in each endosome. For each condition, 10–20 cells were quantified, consisting of intensity measurements from 50–200 vesicles per cell. A similar procedure was followed for the nuclear envelope by measuring the average intensity of a profile traced through the nuclear envelope, identified by the presence of GFP–2FKBP–LBR. Each condition represents an average intensity ratio around the nucleus of 10–20 cells.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

N.J., R.D.B., V.C. and B.L. designed the experiments; N.J. and B.L. prepared the manuscript; N.J., G.H.C.C. and M.G.L. performed the experiments; A.A. provided reagents; all authors reviewed and approved the manuscript prior to submission.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.172775/-/DC1

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