Identification of cAMP-dependent kinase as a third *in vivo* ribosomal protein S6 kinase in pancreatic β-cells

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Ribosomal protein S6 (rpS6) is phosphorylated *in vivo* by isoforms of p70 S6 kinase (S6K) and p90 ribosomal S6 kinase (Rsk) and there is good evidence that it plays a positive role in controlling pancreatic β-cell size and function. In this report, we demonstrate, in the pancreatic β-cell line MIN6 and islets of Langerhans, that agents which stimulate increases in cAMP, such as glucagon like peptide-1 (GLP1) or forskolin, lead to the phosphorylation of rpS6 exclusively at Ser(235/236) independently of the activation of the currently known *in vivo* rpS6 kinases via a pathway that is sensitive to inhibitors of cAMP-dependent kinase (PKA). This cAMP-dependent rpS6 kinase activity is also sensitive to PKI *in vitro* and PKA exclusively phosphorylates recombinant rpS6 on Ser(235/236) *in vitro*. Taken together, we conclude that PKA can phosphorylate rpS6 exclusively at Ser(235/236) *in vivo* in pancreatic β-cells, thus providing a potentially important link between cAMP signalling and the regulation of protein synthesis. Lastly, we provide evidence that PKA is also likely to phosphorylate rpS6 on Ser(235/236) *in vivo* in a number of other mammalian cell types.

Introduction

The Eukaryotic 80S ribosome is a macromolecular complex composed of a small 40S subunit, which decodes the mRNA, and a large 60S subunit, which is involved in peptidyl transfer. In higher eukaryotes, the 40S ribosomal subunit is made up of an 18S RNA and a total of 33 proteins, many of which are phosphorylated [1; 2]. However, of these phosphorylated proteins, ribosomal protein S6 (rpS6) has received the most attention as its phosphorylation is rapidly induced in response to nutritional, hormonal and mitogenic stimuli and it was the first identified substrate for p70S6K (S6K1) [3].

rpS6 is phosphorylated by S6K at the C-terminus on Ser236, Ser235, Ser240, Ser244 and Ser247 *in vitro* [4], which correspond to sites phosphorylated *in vivo* [5]. Deletion of the S6K (dS6K) gene in *Drosophilia* is embryonic lethal; however, a few flies survive and these have reduced body mass as a result of a smaller cell size rather than cell number [6]. Mammalian cells express two isoforms of S6K, S6K1 and S6K2, each encoded by a separate gene [7] and in mice, deletion of the S6K1 gene ($S6K1^{-/-}$) is not lethal but the mice are significantly smaller than their wild type counterparts due to decreased cell size rather than decreased cell number [7 8]. In contrast, mice in which the S6K2 gene is deleted ($S6K2^{-/-}$) show a normal body and cell size compared to wild type littermates [9]. Mice in which both S6K1 and S6K2 ($S6K1^{-/-}/2^{-/-}$) were deleted have a similar phenotype to that of $S6K1^{-/-}$ mice but show a significant decrease in viability compared to the $S6K1^{-/-}$ mice [9]. Importantly, especially in relation to

this study, the phosphorylation of rpS6 appears to play a particularly significant role in controlling pancreatic β-cell size and function as S6K1$^{-/-}$ mice and knock-in mouse in which all five phosphorylatable serine residues within rpS6 are substituted to alanines (rpS6$^{P-/-}$) have comparatively smaller pancreatic β-cells, which co-insides with diminished levels of pancreatic insulin, hypoinsulinemia and impaired glucose tolerance [8 10].

S6K2 is the predominant S6 kinase as growth factor induced phosphorylation of rpS6 at Ser(235/236) is reduced and phosphorylation at Ser(240/244) is undetectable in hepatocytes derived from S6K2$^{-/-}$ knock-out mice [9]. In S6K1$^{-/-}$/2$^{-/-}$ knock-out mice, a proportion of rpS6 remains phosphorylated on Ser(235/236), revealing the presence of another *in vivo* rpS6 Kinase [9]. This was identified as p90 ribosomal S6 kinase (Rsk) [9 11], a kinase that had previously been named due to its ability to readily phosphorylate rpS6 *in vitro* [12].

Although extensively studied, the molecular mechanism by which rpS6 phosphorylation exerts its effects has proved enigmatic. It was originally thought to stimulate the recruitment of 5'TOP (5' terminal oligopyrimidine tract) mRNAs onto polysomes [13]. However, the translation of 5'TOP mRNAs is unaffected in hepatocytes from both S6K1$^{-/-}$/2$^{-/-}$ knock-out mice [9] and rpS6$^{P-/-}$ mice [10]. A recent study provided evidence that site specific phosphorylation of rpS6 on Ser(235/236) via Rsk promotes cap-dependent translation by facilitating the formation of pre-initiation translation complexes [11].

The Glucagon like peptide-1 receptor (GLP1R) is a member of the G-protein coupled receptor (GPCR) super-family, which can couple to $G_s\alpha$ containing heterotrimeric G proteins leading to the activation of adenylyl cyclase and the subsequent increase in the production of cAMP [14]. The GLP1R is highly expressed in pancreatic β-cells and is activated by the hormone GLP1, where it poteniates glucose stimulated insulin secretion, increases β-cell mass and improves β-cell function. These properties have made GLP1 the subject of intensive investigation as a potential treatment for type-2 diabetes. Interestingly, GLP1R activation potently stimulates S6K [15]. Therefore, GLP1 may promote increases in β-cell mass, at least in part, through increases in the phosphorylation status of rpS6. For this reason, we set out to investigate the signalling mechanism by which GLP1 stimulates rpS6 phosphorylation in pancreatic β-cells. In the process, we identified PKA as a third *in vivo* ribosomal kinase which specifically phosphorylates rpS6 on Ser(235/236) in β-cells and in a number of other cell types tested.

Results

Inhibition of phosphoinositide 3-kinase reveals that GLP1 and forskolin can stimulate the phosphorylation of rpS6 on Ser(235/236) via a S6K independent mechanism. In order to investigate the role of phosphoinositide 3-kinase **(**PI3K) in GLP1 stimulated phosphorylation of rpS6 in pancreatic β-cells, MIN6 cells, a pancreatic clonal β-cell line [16], were incubated with glucose, GLP1 or forskolin (an activator of adenylyl cyclase) in the presence or absence of the PI3K inhibitors LY294002 or wortmannin. Glucose, GLP1 and forskolin led to a significant increase in the phosphorylation of S6K, as determined by a shift in its mobility, the phosphorylation of rpS6 on Ser(235/236) and Ser(240/244) and the phosphorylation of PKB on Ser473 (figure 1a-c). Both LY294002 and wortmannin blocked glucose, GLP1 or forskolin stimulated phosphorylation of PKB, S6K1 and rpS6 on Ser(240/244). Glucose stimulated phosphorylation of rpS6 on Ser(235/236) was also blocked by LY294002 or wortmannin (figure 1a). Surprisingly, however, GLP1 and forskolin stimulated phosphorylation of rpS6 on Ser(235/236) was unaffected by the PI3K inhibitors (figures 1b and c). To confirm that GLP1 stimulated phosphorylation of rpS6 at Ser(235/236) was indeed independent of both S6K1 and 2, we determined the activity of S6K1 and 2 in response to GLP1 in the presence and absence of LY294002. GLP1 led to an increase in both S6K1 and 2 activity, which was effectively inhibited by LY294002 (figure 1d and e). Taken together, these results provide evidence that GLP1 and forskolin, agents which elevate cAMP, can stimulate the phosphorylation of rpS6 on Ser(235/236) and Ser(240/244) by two distinct signalling pathways; where the phosphorylation of rpS6 at Ser(240/244) is dependent upon PI3K, likely mediated via the activation of

PKB and S6K, whereas the phosphorylation of rpS6 on Ser(235/236) is mediated independently of PI3K, PKB and S6K1 and 2.

mTORC1 dependent site-specific dephosphorylation of rpS6 on Ser(235/236). To determine whether GLP1 and forskolin stimulated phosphorylation of rpS6 on Ser(235/236) is dependent upon the activation of mTORC1, MIN6 cells were incubated with glucose, glucose plus GLP1 or forskolin in the absence or presence of rapamycin, a specific inhibitor of mTORC1 (figure 2a). Rapamycin blocked glucose stimulated phosphorylation of rpS6 at Ser(240/244) and Ser(235/236), which paralleled the inhibition of S6K1. Rapamycin also blocked GLP1 and forskolin stimulated phosphorylation of rpS6 at Ser(240/244), which again paralleled a decrease in S6K1 phosphorylation and significantly reduced/blocked forskolin and GLP1 stimulated phosphorylation of rpS6 at Ser(235/236). These results indicate that GLP1 and forskolin stimulated rpS6 phosphorylation at Ser(235/236) is dependent upon mTORC1 activity but independent of S6K1 and 2, the only known rpS6 kinases which lies downstream of mTORC1. As anticipated, the phosphorylation of PKB at Ser473 in response to glucose, GLP1 and forskolin was unaffected by rapamycin (figure 2a).
It has been reported that m(TOR) inhibition by nutrient withdrawal or rapamycin can result in the activation of protein phosphatase 2A (PP2A) [17]. Therefore, the rapamycin sensitivity of GLP1 and forskolin stimulated phosphorylation of rpS6 on Ser(235/236) could be attributed to the activation of PP2A. To investigate this possibility, we determined the effect of okadaic acid (a selective inhibitor of protein phosphatases 1 and 2A) and cantharidin (a selective inhibitor of PP2A) (figure 2b) on rapamycin induced inhibition of GLP1 stimulated rpS6 phosphorylation on Ser(235/236). As expected, rapamycin inhibited GLP1 induced rpS6 phosphorylation on Ser(240/244) and Ser(235/236). However, okadaic acid and cantharidin, in the presence of rapamycin, dose dependently stimulated the restoration of GLP1 stimulated rpS6 phosphorylation on Ser(235/236), yet had no effect on the phosphorylation of rpS6 on Ser(240/244) (figures 2b). Collectively, these results provide evidence that inhibition of mTORC1 by rapamycin results in the activation of a phosphatase that could be PP2A, which specifically dephosphorylates rpS6 on Ser(235/236). Therefore, GLP1 stimulated rpS6 phosphorylation on Ser(235/236) is likely to be independent of mTORC1 activation or the activation of its downstream effectors.

Rsk is not responsible for GLP1 stimulated site-specific phosphorylation of rpS6 on Ser(235/236). p90 ribosomal S6 kinase (Rsk) is directly phosphorylated and activated by Erk and can specifically phosphorylate rpS6 on Ser(235/236) via an mTORC1 independent pathway in response to growth factor stimulation [9; 11]. To determine whether GLP1 stimulated site specific phosphorylation of rpS6 on Ser(235/236) is mediated by Rsk, MIN6 cells were incubated with glucose or glucose plus GLP1 in the absence or presence of the MEK inhibitor PD-184352 and its effect on GLP1 stimulated rpS6 phosphorylation assessed (figure 3a). Additionally, in order to investigate selectively the phosphorylation of rpS6 on Ser(235/236), cells were also incubated in the presence and absence of the PI3K inhibitor LY294002 to block signalling through S6K (figure 3a). As observed previously (see figure 1), LY294002 blocked GLP1 stimulated rpS6 phosphorylation on Ser(240/244) and had no significant effect on the phosphorylation of rpS6 on Ser(235/236). LY294002 treatment also stimulated Erk phosphorylation which was effectively inhibited by PD-184352. This LY294002 stimulated Erk activity maybe mediated by LY294002's ability to block K_v currents in pancreatic β-cells [18], which in turn may stimulate Ca^{2+} influx through L-type VGCC, which is sufficient to stimulate Erk activation in β-cells [19]. GLP1 stimulated rpS6 phosphorylation at Ser(235/236) was unaffected by PD-184352 either in the presence or absence of LY294002. Unexpectedly, PD-184352 blocked glucose stimulated rpS6 phosphorylation on Ser(235/236) and Ser(240/244) . However, we found that PD-184352 activated AMPK, as determined by its increased phosphorylation on Thr172 and phosphorylation of a downstream effector acetyl co-carboxylase on Ser79. Increased AMPK activity has been reported to inhibit glucose stimulated mTORC1 signalling and the activation of S6K in β-cells [20]. Indeed, PD-184352, possibly via the activation of AMPK, inhibited mTORC1 as assessed by the phosphorylation status of two downstream targets of mTORC1, 4EBP1 on Ser65 and S6K. Therefore, inhibition of glucose stimulated rpS6 phosphorylation by PD-184352 may be mediated by the activation of AMPK and the inhibition of mTORC1. We also observed that other MEK inhibitors, U0126 and PD-98059, also activated AMPK in MIN6 cells (unpublished results

(Moore and Herbert)). Indeed, it has been previously been reported that both PD98059 and U0126 activate AMPK in HELA and HEK293 cells [21]. Importantly, PD-184352, at a concentration which effectively inhibited GLP1 stimulated Erk phosphorylation, had no observable effect on GLP1 stimulated phosphorylation of rpS6 on Ser235/236. Given that these MEK inhibitors also activate AMPK, which clearly complicates the interpretation of these experiments, we used an alternative approach to inhibit the Erk signalling cascade and hence Rsk. MIN6 cells were infected with recombinant adenovirus encoding Mitogen-activated protein kinase phosphatase 3 (AdMPK3), a dual-specific phosphatase that dephosphorylates the activation loop of ERK1/2 with very high specificity [22], and its effect on GLP1 stimulated rpS6 phosphorylation was assessed (figure 3b). The phorbol ester TPA, which potently activates Erk and Rsk, was used a positive control (figure 3b). Over-expression of MKP3 effectively blocked TPA induced Erk phosphorylation and the phosphorylation of Rsk as assessed by a decrease in Rsk mobility on a SDS-polyacrylamide gel. Yet, overexpression of MKP3 had no effect on GLP1 stimulated rpS6 phosphorylation on either Ser(235/236) or Ser(240/244) in either the presence or absence of LY294002 (figure 3b). Moreover, over expression of MKP3 had no effect on glucose stimulated rpS6 phosphorylation on either Ser(235/236) or Ser(240/244) indicating that the effects of PD-184352 on glucose stimulated phosphorylation of rpS6 observed in figure 3a were likely to be mediated by the activation of AMPK.

Taken together, these results provide strong evidence that Rsk is not responsible for site specific phosphorylation of rpS6 on Ser(235/236) by GLP1 in pancreatic β-cells.

Novel/classical isoforms of PKC are not responsible for GLP1 stimulated site-specific phosphorylation of rpS6 on Ser(235/236). Given that the phosphorylation of rpS6 on Ser(235/236) in response to GLP1 is independent of the known *in vivo* rpS6 kinases, S6K and Rsk, we sought to identify possible candidate kinases that could phosphorylate rpS6 on Ser(235/236) using a program which recognises conserved kinase recognition motifs within proteins (group-based phosphorylation site predicting and scoring platform) [23]. Several kinases that could potentially phosphorylate rpS6 at Ser(235/236) were identified. These included the known S6 kinases S6K and Rsk, as well as PKB, PKC and cAMP-dependent kinase (PKA). However, we have already ruled out the possibility that S6K or Rsk is responsible for GLP1 stimulated rpS6 phosphorylation on Ser(235/236). PKB was also ruled out as GLP1 stimulated phosphorylation of PKB on Ser473 is completely blocked by LY294002 yet the phosphorylation of rpS6 on Ser(235/236) is unaffected (figure 1). To investigate whether PKC is responsible for GLP1 stimulated rpS6 phosphorylation on Ser(235/236), MIN6 cells were treated with GLP1 in the presence or absence of BIM, Ro320432 or Gö6976, selective inhibitors of the novel/classical isoforms of PKC (PKCn/c) (figure 4). GLP1 was also added to MIN6 cells chronically pre-stimulated with TPA to down-regulate both PKCn/c isoforms (figure 4). To selectively investigate the phosphorylation of rpS6 on Ser(235/236), cells were also incubated in the presence and absence of the PI3K inhibitor LY294002 to block signalling through S6K. TPA, which acts via the activation of both PKCn/c, was used as a positive control. As anticipated, chronic pre-stimulation of cells with TPA or addition of BIM, Ro320432 and Gö6976 blocked TPA induced phosphorylation of Erk1/2 and rpS6 yet had no effect on GLP1 stimulated rpS6 phosphorylation on Ser(235/236) either in the presence or absence of LY294002. As shown previously (figure 1), LY294002 effectively blocked GLP1 signalling to rpS6 on Ser(240/244). These results demonstrate that neither classical or novel isoforms of PKC are responsible for GLP1 stimulated rpS6 phosphorylation on Ser(235/236).

cAMP-dependent kinase (PKA) selectively phosphorylates rpS6 on Ser(235/236) in vitro. Having ruled out the possibility that S6K, Rsk, PKC and PKB are responsible for GLP1 stimulated rpS6 phosphorylation on Ser(235/236) (figures 1, 3 and 4), we investigated whether PKA was responsible for GLP1 stimulated selective phosphorylation of rpS6 on Ser(235/236). Indeed, PKA appeared a likely candidate as PKA is activated by agents that elevate cAMP, such as GLP1 and forskolin, and PKA was predicted to selectively phosphorylate rpS6 on Ser(235/236) *in silico*. Initially, we investigated whether the recombinant catalytic subunit of PKA (PKAc), or a control active S6K1, could directly phosphorylate rpS6 specifically on Ser(235/236) in an *in vitro* kinase assay using a GST fusion protein containing the C-terminus of rat rpS6 as substrate (GST-S6). PKAc readily stimulated the incorporation of ^{32}P into GST-S6 but not into a mutant rpS6 protein in which Ser(235)

and Ser(236) were replaced by alanines (GST-S6AA), indicating that PKA exclusively phosphorylates rpS6 on Ser(235/236) (figure 5ai and iii). The stoichiometry of PKA-induced phosphorylation of GST-S6 *in vitro* was determined to be 1.74mol/mol. In contrast, S6K could phosphorylate GST-S6 and GST-S6AA (figure 5ai). It has been reported that the phosphorylation of rpS6 on Ser(235/236) precedes the phosphorylation of Ser(240/244) [24]. Therefore, the phosphorylation of rpS6 on Ser(235/236) may be required for its subsequent phosphorylation on Ser(240/244). In order to rule out this possibility, we also investigated if PKAc could stimulate the incorporation of ^{32}P into a mutant of rpS6 where Ser(235/236) were replaced with phospho-mimetic aspartic acid residues (GST-S6DD). However, neither PKAc or S6K1 was able to phosphorylate GST-S6DD (unpublished results) indicating that perhaps the aspartic acids were unable to act as a true phosphomimetic. Therefore, we also looked at the phosphorylation of GST-S6 in vitro by PKAc or S6K1 using phospho-specific antibodies. Phosphorylation of GST-S6 by PKAc was readily detectable at Ser(235/236) but not at Ser(240/244) (figure 5b). Therefore, PKA selectively phosphorylate rpS6 on Ser(235/236) *in vitro*. In contrast, S6K1 phosphorylation of GST-S6 was detectable at both Ser(235/236) and Ser(240/244). These results are in agreement with a previous report in which the sites of PKA phosphorylation of rpS6 *in vitro,* using purified ribosomes from rats as substrate, were mapped to Ser235 and Ser236 [25].

Evidence that PKA phosphorylates rpS6 on Ser(235/236) in vivo. Agents that lead to an increase in cAMP also lead to the site specific phosphorylation of rpS6 on Ser(235/236) via a S6K, Rsk and PKCn/c independent mechanism. Additionally, we show that PKA, which is activated by elevated levels of cAMP, effectively and selectively phosphoylates rpS6 on Ser(235/236) *in vitro.* To determine whether GLP1 and forskolin treatment resulted in the activation of an *in vitro* rpS6 kinase activity, rpS6 kinase activity was assayed, using either recombinant GST-S6 or GST-S6AA as substrates, in lysates from MIN6 cells treated with GLP1 or forskolin in either the absence or presence of LY294002 and PD-184352 to inhibit both S6K and Rsk activity (figure 6a). Both GLP1 and forskolin treatment led to an increase in S6 kinase activity compared to control lysates as determined by increased ^{32}P-incorporation into GST-S6. This activity was also undiminished in lysates from cells treated by LY294002 and PD-184352. However, the addition of the PKA inhibitor PKI effectively blocked this *in vitro* rpS6 kinase activity, demonstrating that PKA is the major *in vitro* S6 kinase found in cell lysates treated with either GLP1 or forskolin. Moreover, GLP1 or forskolin was unable to stimulate the incorporation of ^{32}P into GST-S6AA, demonstrating that this S6 kinase activity is directed solely towards Ser(235/236).

To provide further evidence that PKA is an *in vivo* rpS6 kinase MIN6 cells were treated with GLP1 or forskolin in the presence or absence of the cell permeable selective adenylyl cyclase inhibitor MDL12,330A and PKA inhibitor H89 (figure 6b and c). Both MDL12,330A and H89 inhibited GLP1 and forskolin stimulated rpS6 phosphorylation. In addition, cells were treated with a penetratin linked cell permeable PKI peptide (PKI_{RR}) that specifically inhibits PKA or, as a negative control, a penetratin linked PKI peptide in which 2 arginine residues, critical for binding of PKI to PKA [26], were replaced by alanines (PKI_{AA}) (figure 6d). PKI_{RR} but not PKI_{AA} effectively inhibited forskolin stimulated rpS6 phosphorylation. PKI_{RR} but not PKA_{AA} also inhibited GLP1 stimulated phosphorylation of rpS6 on Ser(235/236) in the absence or presence of the PI3K inhibitor LY29002. This data, along with the results presented in figures 1 to 5, provide strong evidence that PKA acts as an *in vivo* rpS6 kinase.

Evidence that PKA phosphorylates rpS6 on Ser(235/236) in islets of Langerhans. In order to provide evidence that PKA is able to phosphorylate rpS6 in primary β-cells, rat islets of Langerhans were incubated with glucose, glucose plus GLP1 or forskolin in the presence or absence of the PI3K inhibitors LY294002 or wortmannin (figure 7a), the MEK inhibitor PD-184352 (figure 7b) or the mTORC1 inhibitor rapamycin (figure 7c). Glucose, glucose plus GLP1 and forskolin all led to increases in the phosphorylation of rpS6 (figure 7). Glucose and glucose plus GLP1 also led to increases in PKB phosphorylation (figure 7a). Glucose stimulated phosphorylation of rpS6 and PKB was inhibited by LY294002 and wortmannin (figure 7a). GLP1 and forskolin stimulated

phosphorylation of rpS6 on Ser(240/244) was also blocked by LY294002 and wortmannin as was GLP1 stimulated PKB phosphorylation (figure 7a). However, GLP1 and forskolin stimulated phosphorylation of rpS6 on Ser(235/236) was resistant to both LY294002 and wortmannin indicating that cAMP-dependent phosphorylation of rpS6 on Ser(235/236) is independent of S6K in islets (figure 7a). GLP1 stimulated phosphorylation of rpS6 on Ser(235/236) was also resistant to PD-184352 at a concentration that effectively inhibited TPA induced phosphorylation of Erk, indicating that phosphorylation of this site is also independent of Rsk in islets (figure 7b). To provide evidence that PKA is an *in vivo* rpS6 kinase in islets, islets were treated with GLP1 in the presence or absence of the selective PKA inhibitor H89 (figure 7c). H89 inhibited GLP1 stimulated rpS6 phosphorylation on Ser(235/236) and Ser(240/244). H89 had no effect on the phosphorylation of S6K1 on Thr389. Taken together, these results provide evidence that Ser(235/236) is being phosphorylated by a PKA dependent mechanism. Similar to the results obtained in MIN6 cells. Rapamycin treatment inhibited glucose, GLP1 or forskolin stimulated rpS6 phosphorylation on all sites investigated (figure 7d) , presumably through the activation of a phosphatase (see figure 2). These results provide evidence that the phosphorylation of rpS6 in islets by agents which elevate cAMP occurs via a similar, if not identical, mechanism to that observed in MIN6 cells.

Evidence that PKA phosphorylates rpS6 on Ser(235/236) in other cell types. In order to investigate whether PKA is also able to phosphorylate rpS6 on Ser(235/236) in other cell types, the human fibroblast cell line NIH3T3, the rat pheochromocytoma cell line PC12, the human neuroblastoma cell line SY-SH5Y, the human embryonic kidney cell line HEK293 and the Chinese Hamster Ovarian (CHO) cell line, and as control MIN6 cells, were treated with Forskolin and IBMX to elevate cAMP in the absence or presence of H89, LY294002, LY294002 plus PD184352, or rapamycin (figure 8). In all cell types tested Forskolin and IBMX stimulated the phosphorylation of rpS6 on Ser(235/236) and this was blocked by H89. Importantly, the phosphorylation of rpS6 on Ser(235/236) was resistant to LY294002 or LY294002 plus PD184352 in MIN6 and HEK293 cells and partially resistant in PC12, SY-SH5Y, and NIH3T3 cells. In addition, in HEK293, PC12, SY-SH5Y, and NIH3T3 cells the phosphorylation of rpS6 at Ser(235/236) phosphorylation was also resistant to rapamycin. This demonstrates that, in MIN6, HEK293, PC12, SY-SH5Y, and NIH3T3, increases in cAMP can stimulate the phosphorylation of rpS6 on Ser(235/236) via a Rsk and S6K independent mechanism, which is sensitive to H89. Therefore, based on these results and the results presented throughout the paper, rpS6 can be phosphorylated on Ser(235/236) by PKA in a number of different cell types. The phosphorylation of rpS6 on Ser(240/244) was blocked by LY294002, indicating that the phosphorylation of this site is dependent on PI3K and possibly mediated by S6K. In CHO cells the phosphorylation of rpS6 on Ser(235/236) was also blocked by LY294002 indicating that in this cell type cAMP induced phosphorylation of rpS6 is likely mediated by S6K. .

Discussion

rpS6 was shown to be phosphorylated at multiple sites in response to partially hepatoctomy in rats, a treatment which leads to the rapid increase in protein synthesis [27]. The sites phosphorylated on rpS6 *in vivo* were mapped to the C-terminus and identified as Ser235, Ser236, Ser240, Ser244 and Ser247 [5; 28; 29]. Although a number of protein kinases were shown to phosphorylate rpS6 *in vitro*, including protease-activated kinases, PKC and PKA, these were unable to phosphorylate rpS6 at all the sites identified *in vivo* and their activation *in vivo* did not precede increases in rpS6 phosphorylation (reviewed by [1; 30]). However, a predominant mitogen activated S6 kinase activity, which could phosphorylate rpS6 on all 5 sites identified *in vivo*, and whose activity preceeded the phosphorylation of rpS6 *in vivo*, was isolated and named p70S6 kinase (S6K1) [4 30]. In addition, inhibition of mTORC1, an upstream activator of S6K1/2, inhibited the phosphorylation of rpS6 *in vivo* [7; 31; 32]. Therefore, S6K(1/2) was considered to be the only authentic *in vivo* rpS6 kinase. Examination of the phosphorylation status of rpS6 in hepatocytes from S6K1/2$^{-/-}$ mice demonstrated that S6K is the predominant rpS6 kinase but also revealed the presence of another *in vivo* kinase which: 1.) could exclusively only phosphorylate rpS6 on Ser(235/236); 2.) was sensitive to inhibitors of MEK; and 3.) was insensitive to rapamycin [9]. This kinase was identified as Rsk, a serine/threonine kinase that is

activated in response to mitogens such as EGF and Serum [11]. However, the possibility remained that other *in vivo* rpS6 kinases existed as rpS6 phosphorylation had only been studied in response to a limited number of mitogens, none of which stimulate increases in cAMP, in hepatocytes derived from S6K1/2$^{-/-}$ mice and S6K1/2$^{-/-}$ MEFs [9]. In this study, we identify PKA as a third *in vivo* rpS6 kinase and show that this kinase exclusively phosphorylates rpS6 on Ser(235/236). Interestingly, there are numerous reports demonstrating that agents which elevate cAMP lead to an increase in the phosphorylation of rpS6. However, increased cAMP can activate PKB [33; 34; 35; 36], which in turn can stimulate S6K1/2 via the inactivation of Tsc1/2 and the subsequent increase in mTORC1 activity [37]. In addition, increases in cAMP can also activate the Erk signalling pathway [38], which can, via Rsk, directly phosphorylate rpS6 [11]. Moreover, the Erk signalling pathway can stimulate the activity of S6K1/2 via the inactivation of the Tsc1/2 and the subsequent activation of mTORC1 [39; 40; 41]. Therefore, prior to this report, cAMP-dependent phosphorylation of rpS6 was likely assumed to be mediated by an increase in the activation of Rsk and/or S6K. Indeed, in all the cell types we tested, cAMP-dependent increases in the phosphorylation of rpS6 at Ser(240/244) were blocked by the PI3K inhibitor LY294002, indicating that phosphorylation at these sites is likely mediated by S6K; however, in most cases, cAMP stimulated increases in the phosphorylation of rpS6 on Ser(235/236) was resistant to inhibitors of PI3K or MEK but sensitive to H89.Therefore, we believe that cAMP-dependent phosphorylation of rpS6 on Ser(235/236) in pancreatic β-cells, as well as in many other cell types, is mediated by PKA *in vivo*.

Agents which stimulate increases in cAMP can either inhibit or stimulate protein synthesis depending upon physiological stimuli and cell type. In β-cells, the GLP1R agonist exendin-4 stimulates protein synthesis via a cAMP-dependent mechanism [42]. This is possibly facilitated by PKA-dependent phosphorylation of rpS6, as site specific phosphorylation at Ser(235/236) has previously been shown to stimulate preinitiation translation complex assembly and the initiation of cap-dependent translation [11]. However, polysomes from livers of rpS6$^{p-/-}$ mice are similar to those found in WT mice and, unexpectedly, the rates of protein synthesis are higher in MEFs derived from rpS6$^{p-/-}$ mice compared to those derived from WT mice [10]. Therefore, it is far from clear what role rpS6 phosphorylation plays in the regulation of general protein synthesis. GLP1 also stimulates β-cell proliferation, growth, differentiation and inhibits apoptosis [14]. Therefore, it is tempting to speculate that PKA-dependent site specific phosphorylation of rpS6 in response to GLP1 plays an important role in the translational regulation of a specific subset of transcripts involved in one or more of these processes. Indeed, evidence from rpS6$^{p-/-}$ and S6K1$^{-/-}$ mice demonstrates that the phosphorylation of rpS6 plays a particularly important role in the growth and function of the pancreatic β-cell [8; 10]. The β-cells from the rpS6$^{p-/-}$ mice are smaller in size but total β-cell mass is unaffected due to increased β-cell number [10]. Yet both pancreatic insulin content and circulating levels of insulin in rpS6$^{p-/-}$ mice is half of that found in WT mice [10]. Although similar phenotypic changes were observed in the S6K1$^{-/-}$ mice, decreases in insulin were attributed to a decrease in β-cell mass/volume [8]. As cAMP signalling to rpS6 is unaffected in S6K1$^{-/-}$ mice, PKA-dependent phosphorylation of rpS6 cannot compensate for the loss of S6K1. However, PKA dependent phosphorylation of rpS6 may still play a role in stimulating increases in β-cell mass in response to specific cues. Moreover, the volume of individual β-cells from rpS6$^{p-/-}$ mice is 35% smaller than its wild type counterpart [10], whereas the volume of individual β-cells from S6K1$^{-/-}$ mice is 24% smaller than its wild type counterpart [8]. Unfortunately, there is no data available on the volume of β-cells from S6K1$^{-/-}$/2$^{-/-}$ mice but β-cell size is unaffected in S6K2$^{-/-}$ mice. Therefore, the increased β-cell volume observed in S6K1$^{-/-}$ mice compared to the β-cells from rpS6$^{p-/-}$ may be due to the maintained phosphorylation of rpS6 on Ser(235/236), possibly mediated by PKA. However, it is as well conceivable that PKA-dependent phosphorylation of rpS6 may play a role distinct from translation as a number of ribosomal proteins have been shown to have extra ribosomal functions [43].

PP2A is a major Ser/Thr phosphatase consisting of a catalytic (c) and scaffolding (A) subunit (reviewed in [17]). It's substrate specificity and subcellular localization is mediated by regulatory B subunits. In yeast, Tap42, when phosphorylated by TOR, binds to and inhibits PP2Ac. Rapamycin treatment causes dissociation of this complex, resulting in PP2Ac directing its activity towards downstream targets of TOR. In mammalian cells, PP2Ac has been found to associate with a homolog

of Tap42 called α4, also in a rapamycin-sensitive fashion. Moreover, in Jurkat cells, PP2A is activated by rapamycin treatment or nutrient deprivation, resulting in a decrease in the phosphorylation of downstream targets of mTORC1 [44]. PP2A is likely to play an important role in nutrient regulated β-cell function and nutrient secretagogues such as glucose inhibit PP2A in β-cells (reviewed in [45]). Interestingly, we provide evidence that a phosphatase, likely PP2A, is also activated by inhibition of mTORC1 in β-cells. We show that the activation of this phosphatase in response to rapamycin results in the dephosporylation of rpS6 specifically on Ser(235/236). We suggest that the inhibition of mTORC1 caused by a decrease in glucose concentration would activate PP2A and play an important role in β-cell function.

In conclusion, we have identified PKA as a third *in vivo* rpS6 kinase that specifically phosphorylates rpS6 on Ser(235/236) in response to GLP1 in pancreatic β-cells. This is likely to be a paradigm for cAMP dependent signalling to rpS6 in response to other physiological stimuli which induce increases in cAMP not only in β-cells but in other cell types. This work also provides a potentially important link between cAMP-dependent signalling and protein synthesis.

Materials and Methods

Reagents. Foetal calf serum was purchased from Invitrogen. [^{32}P]-γATP was purchased from Amersham Pharmacia Biotech. All other chemicals and reagents were purchased from Sigma unless otherwise stated. Penatratin coupled PKI peptides (PKI-RR:TTYADFIASGRTGRRNAIDHDAARQIKIWFQNRRMKWKK and PKI-AA:TTYADFIASGRTGAANAIDHDAARQIKIWFQNRRMKWKK) were designed by Professor Chris Proud, University of Southampton, UK and generated by Dr G. Bloomberg, Department of Biochemistry, Bristol university, UK. Adenovirus expressing MKP3 was kindly provided by Professor Phillip Pratt, Department of Pharmacology and Toxicology, Medical College of Wisconsin, USA.

Cell Culture and Treatments. In this study, MIN6 cells (kindly provided by Prof. Jun-Ichi Miyazaki) were used between passages 25-50 at ~80% confluence. MIN6 cells were grown in DMEM containing 25mM glucose supplemented with 15% heat-inactivated foetal calf serum, 100μg/ml streptomycin, 100units/ml penicillin sulphate, 75μM β-mercaptoethanol and 40mM sodium bicarbonate, equilibrated with 5% CO_2, 95% air at 37°C. Prior to treatment, cells were serum starved overnight. The medium was then removed, the cells were washed twice with modified HEPES-balanced Krebs-Ringer bicarbonate buffer (KRB) ((115mM NaCl, 5mM KCl, 10mM $NaHCO_3$, 2.5mM $MgCl_2$, 2.5mM $CaCl_2$, 20mM HEPES, pH7.4 supplemented with 0.5X MEM amino acids solution (50X stock from Sigma), 0.5X MEM non-essential amino acids solution (100X stock from Sigma), and 0.5X L-glutamine (100X stock from Sigma)) and then incubated for 1h at 37°C in KRB prior to incubation in KRB or KRB containing 20mM glucose or 20mM glucose plus 10nM GLP1 for a further hour at 37°C (unless otherwise stated). Full details of treatments are provided in the figure legends. After treatment, the cells were washed with ice cold PBS and then lysed by the addition of ice-cold lysis buffer containing 1% triton, 10mM β-glycerophosphate, 50mM tris-HCl pH7.5, 1mM EDTA, 1mM EGTA, 1mM sodium orthovanadate, 1mM benzamidine-HCl, 0.2mM phenylmethylsulfonyl fluoride, 1μg/ml each of leupeptin and pepstatin, 0.1% β-mercaptoethanol and 50mM sodium fluoride (unless otherwise stated). The lysates were then centrifuged for 10min at 16000 x g. The supernatants were kept and total protein concentrations were determined by the Bradford assay (Bio-Rad). The protein lysates were stored at –80°C until further analysis.

Islet Isolation and Culturing. Pancreatic islets were isolated from 200-250g male Sprague Dawley rats by collagenase digestion and Histopaque density gradient centrifugation as previously described [46]. Islets were cultured for 16h in RPMI 1640 containing 5.6mM glucose, 100 units/ml penicillin and 100μg/ml streptomycin. Prior to treatment, islets were washed twice with KRB buffer minus glucose. The cells were then incubated for 2h at 37°C in KRB buffer containing 2mM glucose prior to incubation in KRB buffer containing 2 or 20mM glucose for the times indicated in the figure legends

(full details of treatments are provided in the figure legends). After treatment, islets were collected by centrifugation for 1min at 200 x *g* and lysed by the addition of ice-cold lysis buffer.

Construction of Plasmids. pGST-S6AA, where Ser235 and Ser236 were changed to alanines, and pGST-S6DD, where Ser235 and Ser236 were changed to aspartic acid, were generated by site-directed mutagenesis using pGST-S6 (a bacterial expression plasmid encoding GST fused to the C-terminal of rat rpS6) as template. Site-Directed mutagenesis was carried out using the QuikChange mutagenesis kit according to the manufacturer's instructions (Stratagene).

Protein Expression. The pGST-S6 constructs were transformed into *Escherichia coli* (strain Rosetta BL21) and grown in Luria-Bertani Broth (LB) at 37^{o}C. Protein production was initiated after the cells had reached OD_{600} of 0.9 by the addition of 1mM isopropyl β-D thiogalactopyranoside (IPTG). After 3h, the cells were collected by centrifugation for 15 minutes at 5000 x *g* at 4˚C. The cells were then resuspended in 2–5 ml of lysis buffer (5% glycerol (v/v), 1M KCl, 20mM Tris HCl pH8.0, 3mM $MgCl_2$, 5mM β-mercaptoethanol, 5mM NaF, 1mM PMSF, leupeptin (1μg/ml), pepstatin (1μg/ml), 0.1% triton) per gram of pelleted cells (wet weight) and then sonicated. Bacterial debris was removed by centrifugation at 16000 x g for 15 minutes at 4˚C. The fusion proteins were then isolated from the supernatants using glutathione Sepharose beads (Amersham) according to the manufacturer's instructions. The proteins were eluted using Elution Buffer (lysis buffer containing 20mM reduced glutathione) and dialysed against: 5% glycerol (v/v), 100mM KCl, 20mM Tris HCl pH7.5, 5mM β-mercaptoethanol, 5mM NaF, 1mM PMSF, 1μg/ml leupeptin, 1μg/ml pepstatin.

S6K1 and S6K2 Kinase Assay. MIN6 cells were lysed in lysis buffer containing 50mM HEPES pH 7.5, 150mM NaCl, 1% (v/v) Nonidet P-40, 2mM EDTA, 50mM sodium fluoride, 10mM sodium pyrophosphate, 1mM sodium orthovanadate, 0.1μg/ml of leupeptin, 0.1μg/ml of pepstatin, 1mM phenylmethylsulfonyl fluoride, and 3mM benzamidine. Whole-cell extracts were centrifuged at 16000 x *g* for 10min at 4°C, and endogeneous S6K1 or S6K2 were immunoprecipitated with the anti-S6K1 or anti-S6K2 antibody immobilized on protein G-Sepharose beads. Immune complexes were washed three times with lysis buffer followed by a single wash with kinase assay buffer (50mM HEPES pH 7.5, 10mM $MgCl_2$, 1mM dithiothreitol, 10mM β-glycerophosphate). The kinase reaction was initiated by resuspending the beads in 25μl of kinase assay buffer supplemented with 1μM PKI (Calbiochem), 50μM ATP, 1μCi of [^{32}P]γATP and 6μg of GST-S6. The reaction was carried out at 30°C for 10min and terminated by the addition of Laemmli sample buffer. Samples were boiled for 5min at 100°C and separated by SDS-PAGE. ^{32}P-labelled GST-S6 visualised by autoradiography. Labelled GST-S6 'bands' were excised from the gel and ^{32}P-incorporation into GST-S6 measured by Cherenkov counting.

cAMP-dependent Kinase Assays Using GST-S6 as Substrate. In vitro phosphorylation of the GST-S6 and mutants by the PKA catalytic subunit (PKAc) was carried out according to the manufacturer's recommendations (Upstate Biotechnology, Inc.). Briefly, reactions containing 3μg GST-S6, 1μCi of [γ-^{32}P]ATP, 5μl ADBI buffer (40mM MOPS pH 7.0, 1mM EDTA) and recombinant PKA diluted in 20mM MOPS pH7.0, 1mM EDTA, 0.01% Brij-35, 5% glycerol, 0.1% β-mercaptoethanol, 1mg/ml BSA were incubated at 30 °C for 10min under constant agitation. The kinase reactions were stopped by addition of Laemmli sample buffer. For rpS6 kinase assays using cell lysates, MIN6 cells were treated as described above and in the figure legends. Kinase reactions containing 20μg cell lysates, 3μg of the recombinant GST-S6 (or GST-S6AA) coupled to GST beads (GE Healthcare), 1 μCi of [^{32}P]-γATP, 12 μM $MgCl_2$, 12 μM Tris-HCl, pH7.5, in the absence or presence of 1 μM PKI, were incubated at 30 °C for 1 h under constant agitation. GST beads were washed twice in lysis buffer prior to the addition of Laemmli sample buffer.

In all cases, samples were boiled for 5min at 100°C and the proteins separated by SDS-PAGE. ^{32}P-labelled GST-S6 was visualised by autoradiography. Labelled GST-S6 'bands' were then excised from the gel and ^{32}P-incorporation into GST-S6 measured by Cherenkov counting.

SDS-PAGE and Western Blotting. SDS-PAGE and Western blotting were performed as described previously [47]. Anti-phospho: Erk Thr202/Tyr204, rpS6 Ser235/Ser236, rpS6 Ser240/Ser244, PKB Ser473, AMPK Thr172, S6K1 Thr389, Acetyl CoA carboxylase (ACC) Ser79, 4EBP1 Ser65 antibodies as well as anti-PKB, Erk2, rpS6, Rsk1, AMPK and S6K1 were purchased from Cell Signalling Technologies, MA, USA. Anti-myc(9E10) antibody was purchased from Sigma. Anti-PKCα, γ and ε antibodies were purchased from Transduction Laboratories. Anti-S6K2 antibody was kindly provided by Professor Ivan Gout (University College London, Institute of Structural and Molecular Biology, London, UK).

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Abbreviations. GLP1, glucagon like peptide 1; MIN6, mouse insulinoma cell line 6; PAGE, polyacrylamide gel electrophoresis; KRB, Krebs-Ringer bicarbonate buffer; S6K, ribosomal protein S6 kinase, rpS6, ribosomal protein S6; Erk, Extracellular regulated kinase, PKB Protein kinase B; PKC, protein Kinase C; PKA, Protein kinase A/cAMP-dependent protein kinase; AMPK, AMP activated protein kinase. Rsk, p90 ribosomal S6 kinase; mTORC1, mammalian target of Rapamycin complex 1;4E-BP, eIF4E binding protein; ACC, Acetyl CoA carboxylase; MKP3, Mitogen-activated protein kinase phosphatase 3; IBMX, isobutylmethylxanthine; PBS, phosphate buffered saline.

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Figure legends

Figure 1: Role of PI3K in rpS6 phosphorylation.
MIN6 cells were preincubated in KRB for 1h. Cells were then treated in KRB for 1h with **a.**) 20mM glucose, **b.)** 20mM glucose plus 10nM GLP1 or **c.**) 10μM forskolin in the presence or absence of LY294002 or wortmannin. Proteins were resolved on SDS-PAGE and Western blotted using antisera against phosphorylated (P) rpS6 on Ser(235/236) (P-rpS6 S235/236) and Ser(240/244) (P-rpS6 S240/244), P-PKB Ser473, S6K1 and, as a loading control, Erk2. **aii., bii, cii**) The effects of 20μM LY294002 or 100nM wortmannin on the phosphorylation of rpS6 on Ser(235/236) were quantified by densitometric analysis. **d. and e.)** MIN6 cells were incubated in KRB for 1h with 20mM glucose plus 10nM GLP1 in the presence or absence of 20μM LY294002. Lysates were subjected to a **d.)** S6K1 and **e.)** S6K2 kinase assay using GST-S6 as substrate and reactions separated by SDS-PAGE and (**di. and ei.)** visualised by autoradiography (top panel) and Coomassie blue staining (bottom panel). **dii. and eii.)** Incorporation of ^{32}P into GST-S6 expressed as percentage of control. All results are representative of independent experiments and shown as means ± SE; $n = 3$. $^{*}P < 0.05$ using a one-way ANOVA. P value obtained using a one-way ANOVA followed by Bonferroni adjustment.

Figure 2: Role of mTORC1 in cAMP-dependent phosphorylation of rpS6.

MIN6 cells were preincubated in KRB for 1 h, and were then treated for a further 1 h in KRB containing glucose 20mM (glu), 20mM glucose plus 10nM GLP1 (GLP1) or 10μM forskolin (Fsk). **a.)** rapamycin (200nM), **b.)** okadaic acid or cantharidin were added 30 min before treatment. Cell lysates were separated on SDS/PAGE and subjected to immunoblotting with antisera against phosphorylated (P) rpS6 Ser(235/236) and Ser(240/244), P-PKB Ser(473), and as loading control total Erk2. (**aii**) Phosphorylation of rpS6 on Ser(235/236) was quantified by densitometric analysis. Results shown as means ± SE; $n = 3$. $*P < 0.05$, $**P < 0.01$. P values obtained using a one-way ANOVA. P value obtained using a one-way ANOVA followed by Bonferroni adjustment. All results are representative of 3 independent experiments.

Figure 3: rpS6 phosphorylation on Ser(235/236) is independent of Rsk.
a.) MIN6 cells were uninfected or (**b.**) infected with the recombinant adenovirus expressing MKP3 (Ad MKP3) for 48h. Cells were preincubated in KRB for 1 h, and were then treated for a further 1h in KRB containing 20mM glucose, 20mM glucose plus 10nM GLP1 or 1μM TPA. 1.25μM PD183452, 20μM LY294002 or both were added 30 min before treatment with glucose and GLP1. Cell lysates were separated on SDS/PAGE and subjected to immunoblotting with antisera against phosphorylated (P) rpS6 Ser(235/236) and Ser(240/244), (P)AMPK Thr(172), (P)Erk, (P)ACC Ser79, (P)4E-BP1 Ser65, Rsk, S6K1 and as loading controls, total rpS6 and AMPK. Quantified data from phospho-rpS6 Ser(235/236) blots is shown below as means ± SE; $n = 4$. No statistical significance was obtained among the samples treated with GLP1 in the presence or absence of PD183452, LY294002 or both. $*P < 0.05$. P value obtained using a one-way ANOVA followed by Bonferroni adjustment.

Figure 4: PKC is not involved in GLP1 mediated rpS6 phosphorylation.
a.) MIN6 cells were preincubated for 1 h in KRB. Cells were then treated for a further 1h in KRB containing 20mM glucose, 20mM glucose plus 10nM GLP1 or 1μM TPA. BIM (1 μM), Ro320432 (1 μM) and Gö6976 (1 μM), or LY294002 (20 μM) were added 30 min before treatment with glucose plus GLP1 or TPA. **a.) and b.)** Cells were also treated for 16h with TPA (O/N TPA). Lysates were separated on SDS-PAGE and Western blotted using antisera specific to phosphorylated (P) rpS6 Ser(235/236) and Ser(240/244), (P)Erk, and to total rpS6 and PKCα, γ, and ε. Results shown are representative of at least three independent experiments.

Figure 5: PKA selectively phosphorylates rpS6 on Ser235/236 *in vitro*.
The PKA activity was determined by *in vitro* kinase assay using GST-S6, or GST-S6AA as substrates and PKAc as enzyme. Samples were resolved by SDS-PAGE. **ai)** Top Panels: autoradiographs of ^{32}P incorporation into substrate Bottom Panels: Gel stained with Coomassie blue. **aii)** ^{32}P incorporation into substrate quantified by Cherenkov counting. Data is presented as means ± SE; $n = 6$ for GST-S6, or 3 for GST-S6AA and GST-S6DD. **b.)** Samples from *in vitro* kinase assays using either PKAc or S6K1 as enzyme and GST-S6 as substrate or, as control, lysates from MIN6 cell treated with GLP1, were separated by SDS-PAGE and Western blotted using antisera against phosphorylated (P) rpS6 (Ser235/236) and Ser(240/244), and total rpS6.

Figure 6: Evidence that PKA phosphorylates rpS6 *in vivo*.
MIN6 cells were pre-incubated in KRB for 1 h. Cells were then treated for a further 1 h in KRB containing: 20mM glucose, 20mM glucose plus 10nM GLP1 or 10μM forskolin. (**a**) 2.5μM PD183452 and 20 μM LY294002 were added 30min before treatment with glucose plus GLP1 or forskolin. The kinase assays were performed on cell lysates (20μg) in the presence or absence of 1μM PKI, using the recombinant proteins GST-S6 and GST-S6AA as substrates. GST-S6 and GST-S6AA were separated by SDS-PAGE Top panel: ^{32}P-incorporation into substrate visualised by autoradiography. Bottom panel: the substrate visualised by Coomassie blue-staining. **b.)** H89, **c.)** MDL12,330A or **d.)** 50μM Pentratin-linked PKI_{RR} or as control PKI_{AA} were added 30min prior to treatment. Proteins were resolved on SDS-PAGE and Western blotted using antisera against phosphorylated (P) rpS6

Ser(235/236) and Ser(240/244), S6K1 and, as loading control, Erk2. All results are representative of at least 3 independent experiments.

Figure 7: Evidence that PKA phosphorylates rpS6 in Islets of Langerhans.
Islets were preincubated in KRB supplemented with 2mM Glucose for 1h. Islets were then treated in KRB for 1h with: 20mM glucose, 20mM glucose plus 10nM GLP1 or 10μM forskolin in the presence or absence of **a.)** LY294002 or wortmannin, **b.)** 1.25μM PD183452 and/or 20μM LY294002, (bottom panel- as a control islets were treated with1μM TPA for 1h in the presence or absence of 1.25μM PD183452) **c.)** 5μM H89 in the presence or absence of 1.25μM PD183452 and 20μM LY294002 or **d.)**200nM Rapamycin. Proteins were resolved on SDS-PAGE and Western blotted using antisera against phosphorylated (P) rpS6 on Ser(235/236) ((P)rpS6 S235/236) and Ser(240/244) ((P)rpS6 S240/244), (P)PKB Ser473, and as loading controls rpS6 or Erk2. Results shown as means ± SE; $n = 3$. $*P < 0.05$, $**P < 0.01$. P value obtained using a one-way ANOVA P value obtained using a one-way ANOVA followed by Bonferroni adjustment. The results presented are representative of 3 independent experiments.

Figure 8: cAMP-dependent phosphorylation of rpS6 in other cell types.
Cells were pre-incubated in KRB for 1 h. Cells were then treated for a further 1h in KRB containing 10 μM forskolin plus 1mM IBMX. 5μM H89, 2.5μM PD183452, 20μM LY294002 or 200nM rapamycin were added 30min before the treatment of forskolin and IBMX. Proteins were resolved on SDS-PAGE and Western blotted using antisera specific to phosphorylated (P) rpS6 Ser(235/236) and Ser(240/244), (p)Erk1/2 and rpS6 as loading control. All results presented are representative of at least 3 independent experiments.

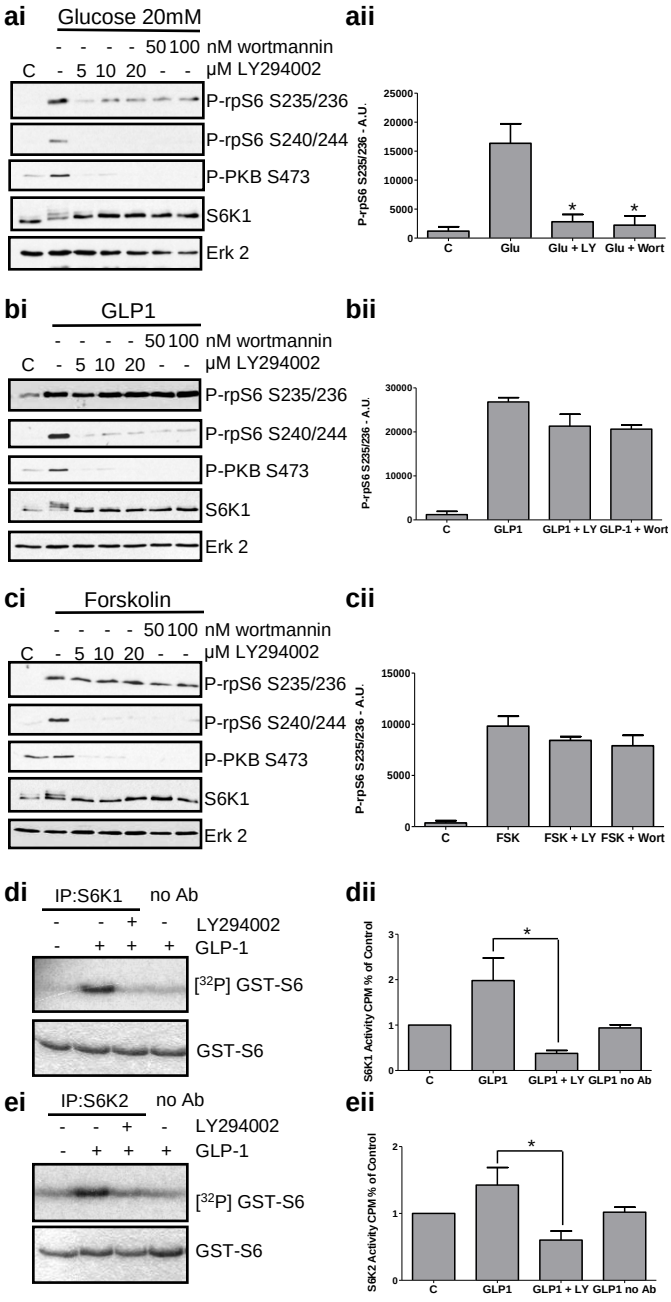
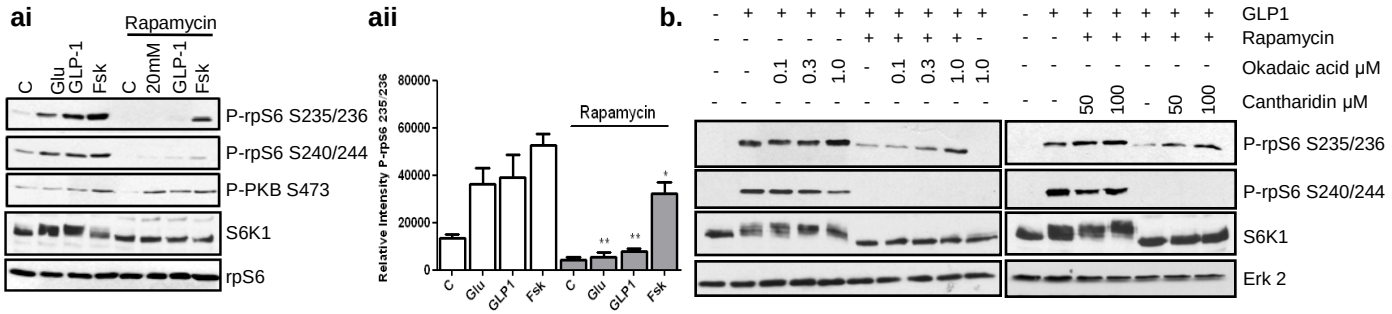

ai
Glucose 20mM
- - - - 50 100 nM wortmannin
C - 5 10 20 - - μM LY294002
P-rpS6 S235/236
P-rpS6 S240/244
P-PKB S473
S6K1
Erk 2
aii
P-rpS6 S235/236 - A.U.
25000
20000
15000
10000
5000
0
C
Glu
Glu + LY
Glu + Wort
bi
GLP1
- - - - 50 100 nM wortmannin
C - 5 10 20 - - μM LY294002
P-rpS6 S235/236
P-rpS6 S240/244
P-PKB S473
S6K1
Erk 2
bii
P-rpS6 S235/236 - A.U.
30000
20000
10000
0
C
GLP1
GLP1 + LY
GLP-1 + Wort
ci
Forskolin
- - - - 50 100 nM wortmannin
C - 5 10 20 - - μM LY294002
P-rpS6 S235/236
P-rpS6 S240/244
P-PKB S473
S6K1
Erk 2
cii
P-rpS6 S235/236 - A.U.
15000
10000
5000
0
C
FSK
FSK + LY
FSK + Wort
di
IP:S6K1
no Ab
- - + - LY294002
- + + + GLP-1
[32P] GST-S6
GST-S6
dii
S6K1 Activity CPM % of Control
3
2
1
0
C
GLP1
GLP1 + LY
GLP1 no Ab
ei
IP:S6K2
no Ab
- - + - LY294002
- + + + GLP-1
[32P] GST-S6
GST-S6
eii
S6K2 Activity CPM % of Control
2
1
0
C
GLP1
GLP1 + LY
GLP1 no Ab

Figure 1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Glu
GLP-1
Fsk
C
20mM
GLP-1
Fsk
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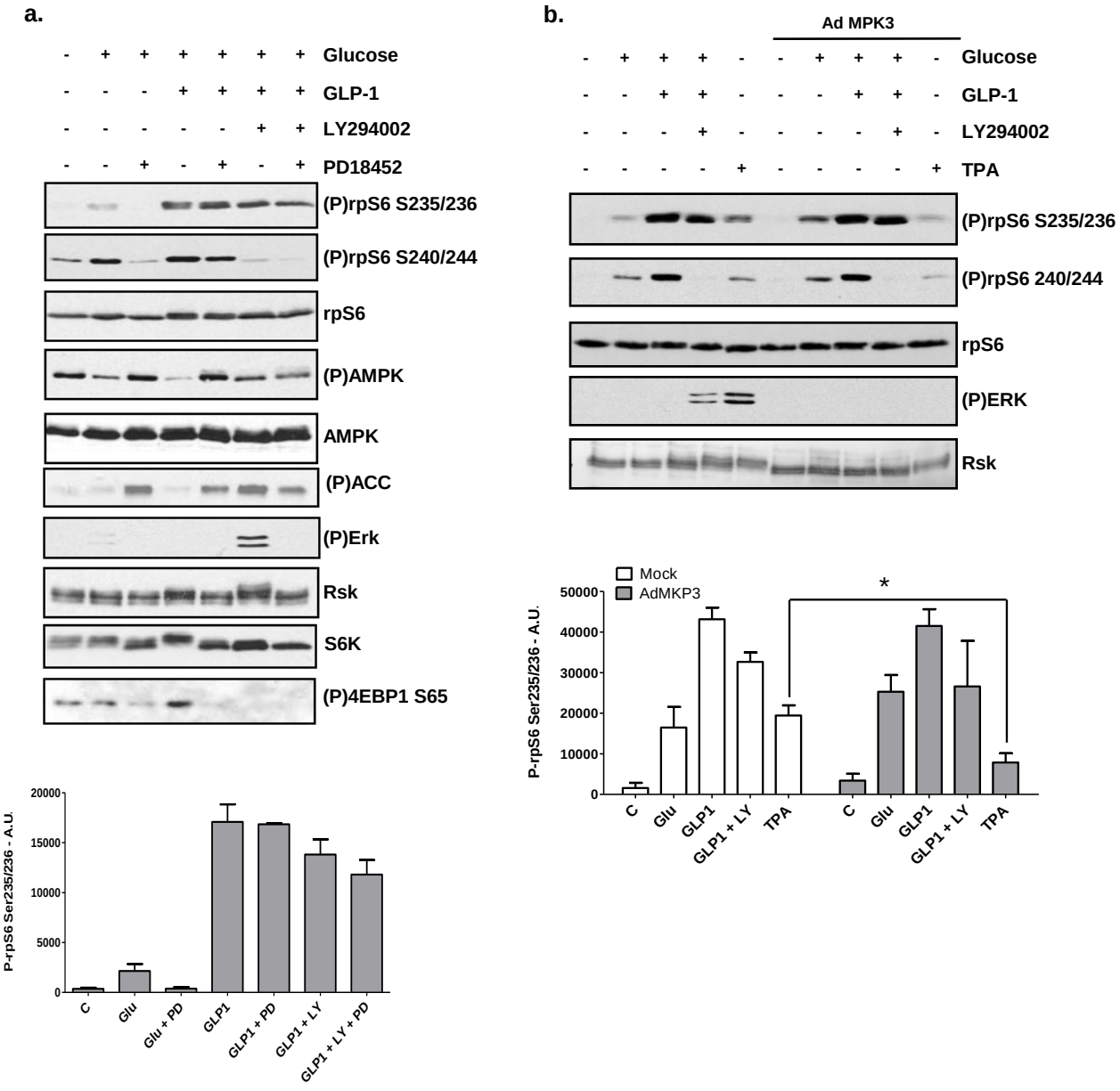
P-rpS6 S240/244
P-PKB S473
S6K1
rpS6
aii
Relative Intensity P-rpS6 235/236
80000
60000
40000
20000
0
Rapamycin
C
Glu
GLP1
Fsk
C
Glu
GLP1
Fsk
b.
GLP1
Rapamycin
Okadaic acid μM
Cantharidin μM
0.1
0.3
1.0
50
100
P-rpS6 S235/236
P-rpS6 S240/244
S6K1
Erk 2

Figure 2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PD18452
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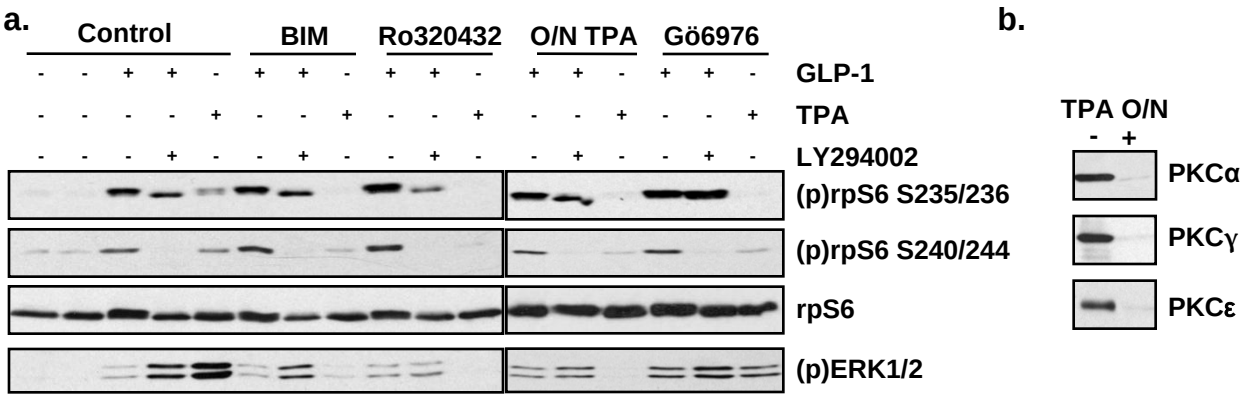
(P)rpS6 S240/244
rpS6
(P)AMPK
AMPK
(P)ACC
(P)Erk
Rsk
S6K
(P)4EBP1 S65
P-rpS6 Ser235/236 - A.U.
C
Glu
Glu + PD
GLP1
GLP1 + PD
GLP1 + LY
GLP1 + LY + PD
b.
Ad MPK3
Glucose
GLP-1
LY294002
TPA
(P)rpS6 S235/236
(P)rpS6 240/244
rpS6
(P)ERK
Rsk
Mock
AdMKP3
*
P-rpS6 Ser235/236 - A.U.
C
Glu
GLP1
GLP1 + LY
TPA

Figure 3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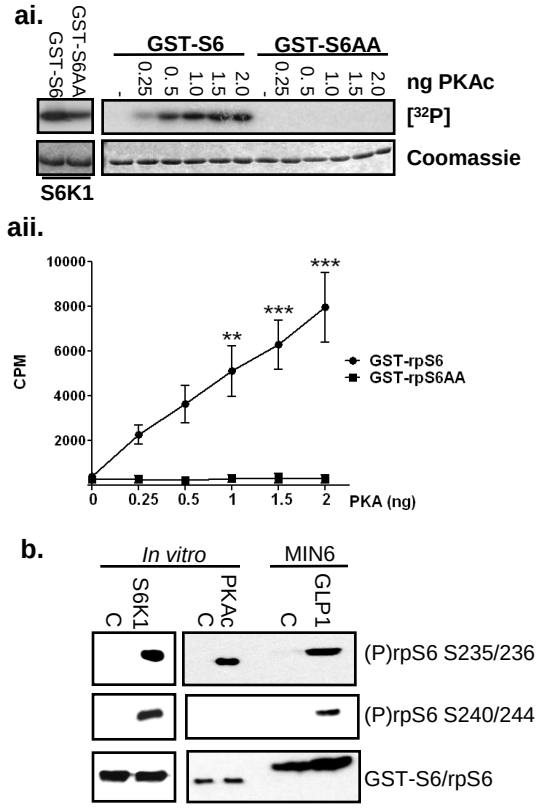
Control
BIM
Ro320432
O/N TPA
Gö6976
GLP-1
TPA
LY294002
(p)rpS6 S235/236
(p)rpS6 S240/244
rpS6
(p)ERK1/2
b.
TPA O/N
- +
PKCα
PKCγ
PKCε

Figure 4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GST-S6AA
GST-S6
GST-S6AA
-
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0. 5
1.0
1.5
2.0
-
0.25
0. 5
1.0
1.5
2.0
ng PKAc
[32P]
Coomassie
S6K1
aii.
CPM
10000
8000
6000
4000
2000
0
0.25
0.5
1
1.5
2
PKA (ng)
**
***
***
GST-rpS6
GST-rpS6AA
b.
In vitro
MIN6
S6K1
PKAc
GLP1
C
C
C
(P)rpS6 S235/236
(P)rpS6 S240/244
GST-S6/rpS6

Figure 5

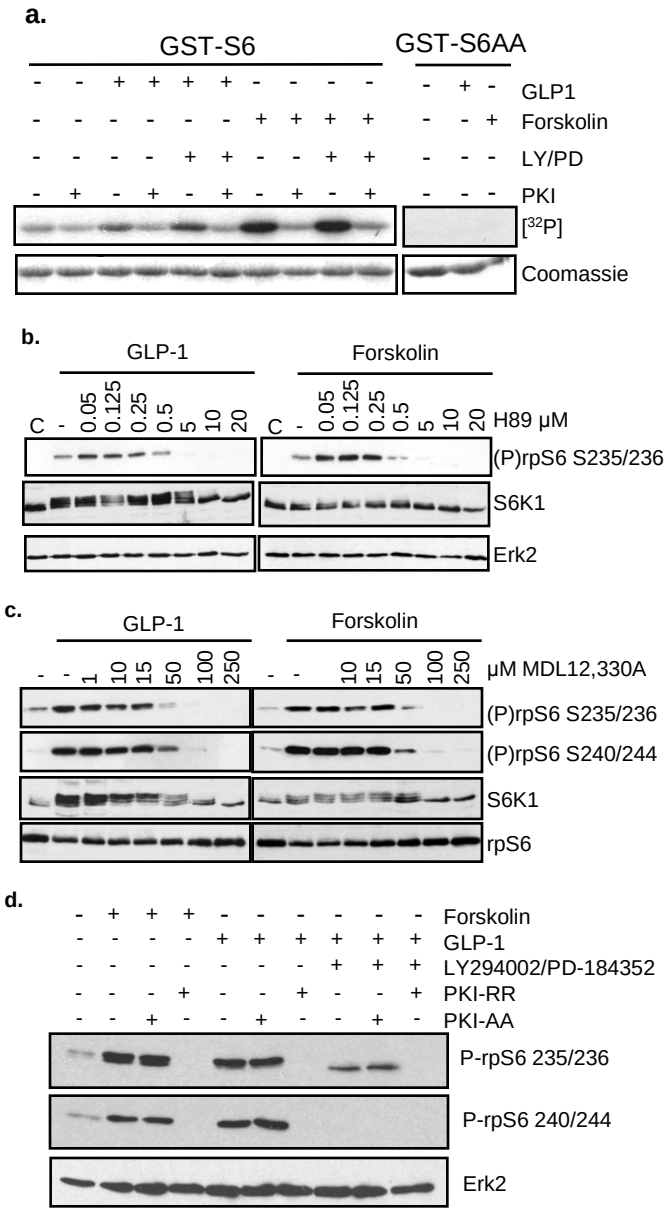
a.
GST-S6
GST-S6AA
GLP1
Forskolin
LY/PD
PKI
[32P]
Coomassie
b.
GLP-1
Forskolin
H89 μM
(P)rpS6 S235/236
S6K1
Erk2
c.
GLP-1
Forskolin
μM MDL12,330A
(P)rpS6 S235/236
(P)rpS6 S240/244
S6K1
rpS6
d.
Forskolin
GLP-1
LY294002/PD-184352
PKI-RR
PKI-AA
P-rpS6 235/236
P-rpS6 240/244
Erk2

Figure 6

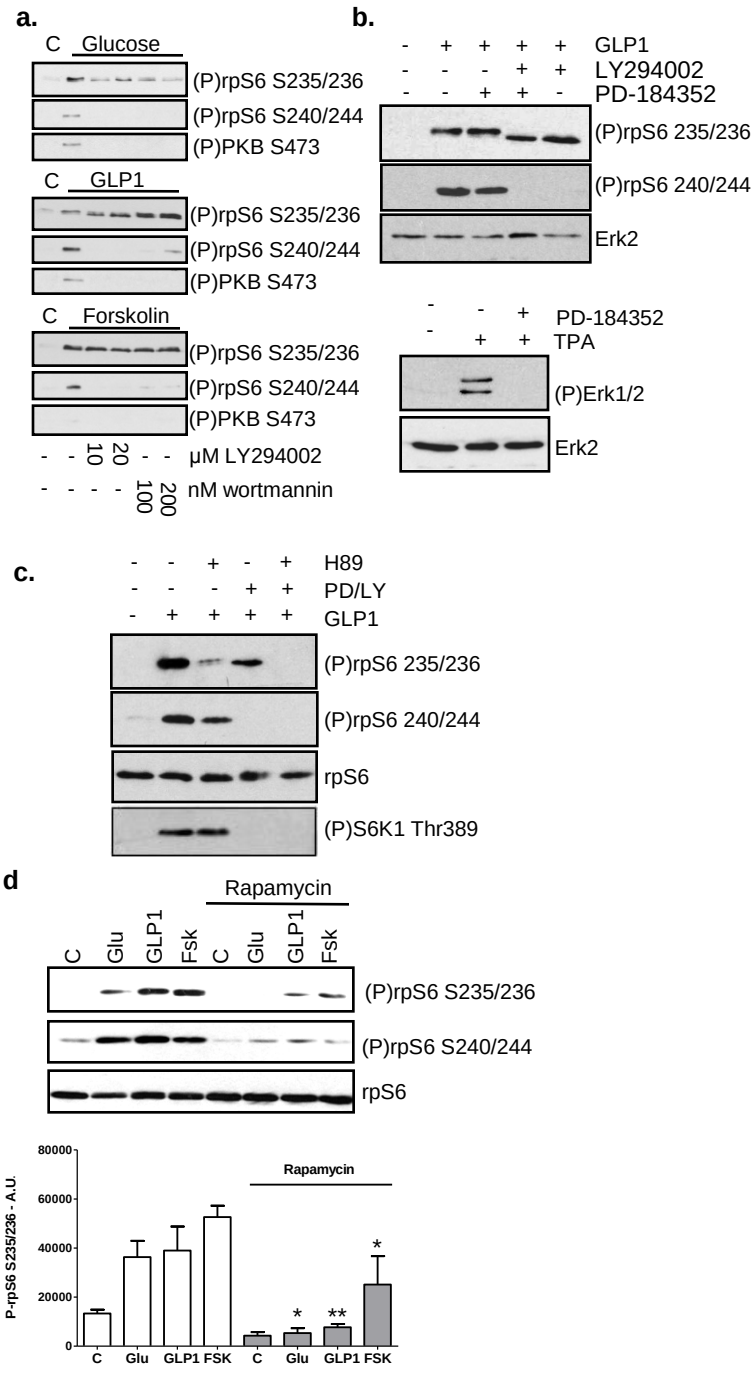
a.
C Glucose
(P)rpS6 S235/236
(P)rpS6 S240/244
(P)PKB S473
C GLP1
(P)rpS6 S235/236
(P)rpS6 S240/244
(P)PKB S473
C Forskolin
(P)rpS6 S235/236
(P)rpS6 S240/244
(P)PKB S473
- - 10 20 - - μM LY294002
- - - - 100 200 nM wortmannin
b.
- + + + + GLP1
- - - + + LY294002
- - + + - PD-184352
(P)rpS6 235/236
(P)rpS6 240/244
Erk2
- - + PD-184352
- + + TPA
(P)Erk1/2
Erk2
c.
- - + - + H89
- - - + + PD/LY
- + + + + GLP1
(P)rpS6 235/236
(P)rpS6 240/244
rpS6
(P)S6K1 Thr389
d
Rapamycin
C Glu GLP1 Fsk C Glu GLP1 Fsk
(P)rpS6 S235/236
(P)rpS6 S240/244
rpS6
P-rpS6 S235/236 - A.U.
80000
60000
40000
20000
0
Rapamycin
C Glu GLP1 FSK C Glu GLP1 FSK
*
*
**
*

Figure 7

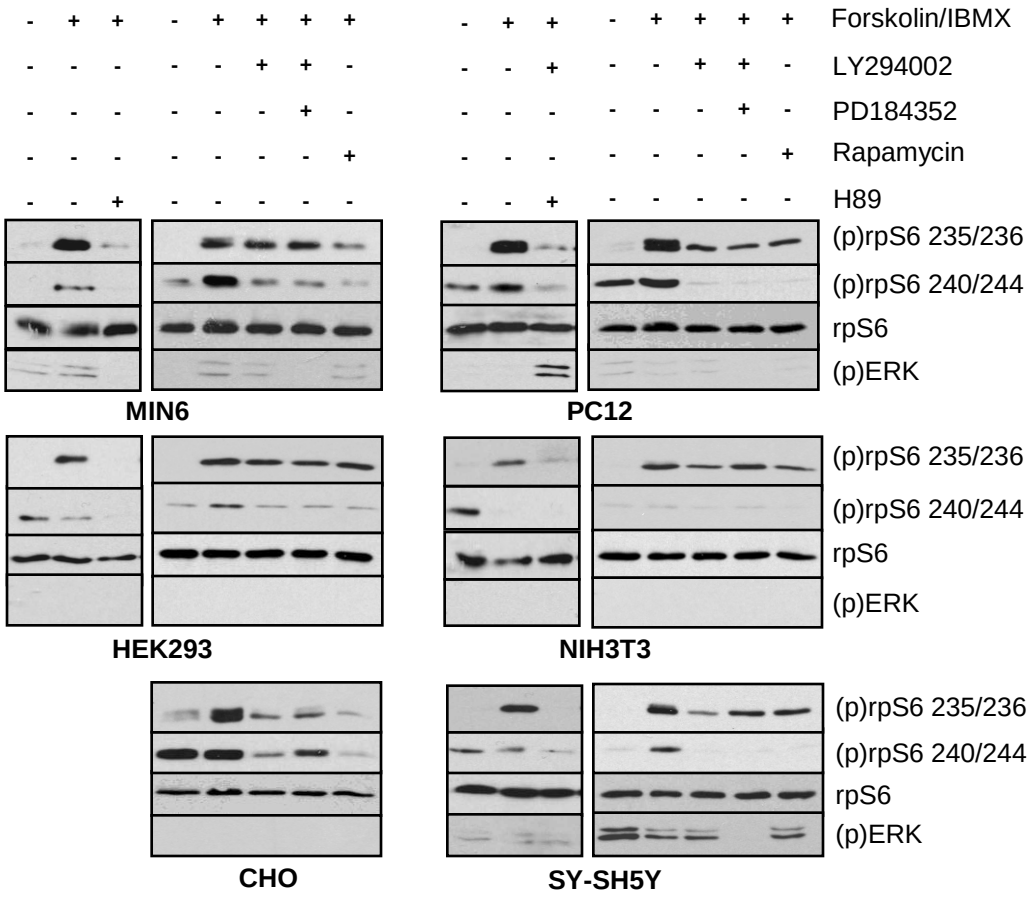
- + + - + + + + - + + - + + + + Forskolin/IBMX
- - - - - + + - - - + - - + + - LY294002
- - - - - - + - - - - - - - + - PD184352
- - - - - - - + - - - - - - - + Rapamycin
- - + - - - - - - - + - - - - - H89
(p)rpS6 235/236
(p)rpS6 240/244
rpS6
(p)ERK
MIN6
PC12
(p)rpS6 235/236
(p)rpS6 240/244
rpS6
(p)ERK
HEK293
NIH3T3
(p)rpS6 235/236
(p)rpS6 240/244
rpS6
(p)ERK
CHO
SY-SH5Y

Figure 8