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Autophosphorylation of serine 608 in the p85 regulatory subunit of wild type or cancer-associated mutants of phosphoinositide 3-kinase does not affect its lipid kinase activity

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Abstract

Background: The α -isoform of the Type 1A Phosphoinositide 3-kinases (PI3K α) has protein kinase activity as well as phosphoinositide lipid kinase activity. The best described substrate for its protein kinase activity is its regulatory subunit, p85 α , which becomes phosphorylated on Serine 608. Phosphorylation of Serine 608 has been reported to down-regulate its lipid kinase activity.

Results: We have assessed whether oncogenic mutants of PI3K α , which have up-regulated lipid kinase activity, have altered levels of Serine 608 phosphorylation compared to wild type PI3K α , and whether differential phosphorylation of Serine 608 contributes to increased activity of oncogenic forms of PI3K α with point mutations in the helical or the kinase domains. Despite markedly increased lipid kinase activity, protein kinase activity was not altered in oncogenic compared to wild type forms of PI3K α . By manipulating levels of phosphorylation of Serine 608 *in vitro*, we found no evidence that the protein kinase activity of PI3K α affects its phosphoinositide lipid kinase activity in either wild-type or oncogenic mutants of PI3K α .

Conclusions: Phosphorylation of p85 α S608 is not a significant regulator of wild-type or oncogenic PI3K α lipid kinase activity.

Keywords: PI3K, PIK3CA, Phosphoinositide, Kinase, Mutation, Oncogene, Phosphorylation

Background

Phosphoinositide 3-kinases (PI3Ks) are a ubiquitous family of lipid kinases that catalyse the phosphorylation of phosphoinositide lipids at the 3' position on the inositide ring [1]. The Class 1A sub-group exists as heterodimers of a p110 catalytic subunit and a p85 regulatory subunit that can phosphorylate three phosphoinositides (PI, PI-(4)-P and PI-(4,5)-P₂) *in vitro*, but are thought to primarily phosphorylate PI-(4,5)-P₂ *in vivo* to form PI-(3,4,5)-P₃ (PIP₃) [1]. PIP₃ resides almost exclusively in the plasma membrane and propagates signals by recruiting and

activating a variety of downstream proteins that contain PIP₃ binding domains (primarily Pleckstrin Homology (PH) domains) [2]. PI3K/PIP₃ signalling regulates a wide range of cellular processes including cell growth, survival, glucose metabolism and migration [1,3].

The prototypic Class 1A PI3K is a heterodimer of the α isoforms of p110 and p85 (p110 α /p85 α or PI3K α). Mutations in *PIK3CA*, the gene encoding p110 α , are oncogenic [4-7] and are frequently reported in breast and colon cancers, where they are found in around 25% of human tumours [8-11]. Tumour-associated *PIK3CA* mutations are all somatic, mono-allelic single base changes that result in single amino acid substitutions. The majority (>80%) of mutations cluster in exon 9 (which codes for the helical domain) or exon 20 (which codes for the kinase domain), most commonly E542K

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and E545K in exon 9 and H1047R in exon 20 [4,12]. Cancer-associated, mutated forms of PI3K α are associated with increased phosphoinositide kinase activity [4-6,13], leading to up-regulation of downstream signalling events such as phosphorylation of Akt and S6 [5,14].

Class 1A PI3Ks also have protein kinase activity. The p110 α catalytic subunit can phosphorylate its regulatory subunit, p85 α , at Serine 608 (S608). Phosphorylation of this site has been reported to result in feed-back inhibition by down-modulating the lipid kinase activity of p110 α [15-17], however the role of phosphorylation of S608 in signalling by endogenous PI3K α and the structural mechanism of down-modulation of lipid kinase activity by S608 phosphorylation are not well described. We have assessed the possibility that the increased lipid kinase activities of oncogenic mutants of PI3K α could be partly due to alterations in the phosphorylation of S608. Rather than make point mutations in S608, which can potentially subtly alter the structure and thus the activity of p110 α /p85 α heterodimers, we have manipulated the levels of phosphorylation of S608 of highly-purified, recombinant p110 α /p85 α *in vitro* and tested the effect on lipid kinase activity. Neither complete dephosphorylation nor a high percentage occupancy of S608 by a phosphate group significantly altered the lipid kinase activity of wild-type PI3K α . The levels and kinetics of S608 phosphorylation in two oncogenic mutants, E545K and H1047R, were not significantly different to that of wild-type p110 α /p85 α and the lipid kinase activities of mutant PI3K α were similarly unaffected by phosphorylation of S608. This suggests that phosphorylation of S608 is not a significant regulator of PI3K α lipid kinase activity.

Results

Expression, purification and characterisation of recombinant wild-type and mutant PI3K α

The same strategy that was used to express and purify active, recombinant bovine p110 α /p85 α [18] was used to generate human, C-terminally EE-tagged, full length, wild-type p110 α /p85 α in Sf9 insect cells using the Bac-N-Blue baculovirus system. When Sf9 cells were co-infected with baculoviruses encoding both subunits, p85 was expressed at higher levels than p110 α EE, therefore to obtain purified p110 α EE/p85 α , affinity chromatography using an antibody directed against the epitope tag (EE mAb) was used to capture the enzyme complex but not excess p85 α . Competitive elution with an EE-tag peptide (EYMPME), followed by anion exchange chromatography were performed as described [18]. PI3K purified in this way comprises a homogeneous 1:1 complex of the p110 α and p85 α subunits as assessed by both SDS-PAGE (Figure 1A) and analytical size exclusion chromatography [18]. Recombinant p110 α EE/p85 α was estimated to be > 95% pure based on densitometry of the relative levels of all Coomassie blue-stained bands in each lane and no peptides other than those derived from p110 α EE or p85 α were detected by LC-MS/MS of tryptic digests of the purified recombinant complex. We have also expressed and purified to homogeneity EE-tagged complexes of p85 α with p110 α containing two of the most common cancer-associated mutations from two different regions of p110 α (E545K in exon 9 and H1047R in exon 20) (Figure 1A).

Oncogenic mutants of PI3K α have been reported to have higher lipid kinase activity than wild-type [4-6,13].

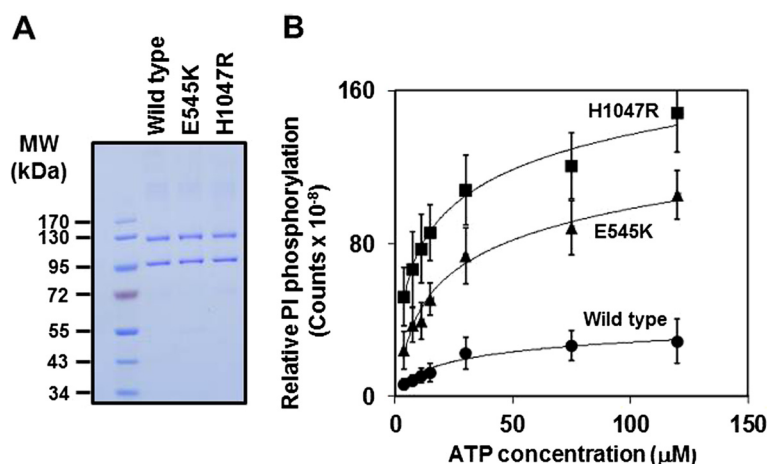


Figure 1 Characterisation of purified, recombinant PI3Ks. **A.** SDS-PAGE and Coomassie Blue staining of molecular weight standards (Fermentas, Lane 1), 0.5 μg purified p110 α EE^{WT}/p85 α (lane 2), 0.5 μg purified p110 α EE^{E545K}/p85 α (lane 3) and 0.5 μg purified p110 α EE^{H1047R}/p85 α (lane 4). **B.** Purified, recombinant PI3Ks were assayed for lipid kinase activity with increasing concentrations of ATP using PI-(4,5)-P₂ as a substrate. Reactions were stopped after 20 minutes using 1 M HCl. The amount of ³²P-PI-(3,4,5)-P₃ generated was quantified using a phosphorimager and displayed as mean ± SEM, n = 4.

As shown previously [15,19], recombinant forms of the p110αEE^{E545K}/p85α and p110αEE^{H1047R}/p85α complexes have higher lipid kinase activities than p110αEE^{WT}/p85α. The H1047R mutant had higher activity than the E545K mutant (Figure 1B). Kinetic parameters were derived for recombinant wild type and mutant p110αEE/p85α complexes by varying the concentration of ATP and measuring initial reaction rates (Table 1), and showed that, when PI was used as the substrate, the K_m for H1047R and E545K were similar to wild type. When PI-(4,5)-P₂ was used as a substrate, the K_m for H1047R was decreased approximately 2-fold compared to wild type, but that for E545K was not significantly different. Both mutants showed a significant 3 to 4-fold increase in V_{max} when either PI or PI-(4,5)-P₂ was used as a substrate, with the V_{max} for H1047R being significantly higher than that for E545K (Table 1). The kinetic characteristics of purified, recombinant, EE-tagged forms of wild-type and mutant PI3Kα are therefore essentially the same as those previously reported for purified, recombinant His-tagged forms of PI3Kα [15,19] and endogenous wild-type and mutant PI3K [4,5].

Effect of dephosphorylation of p85α S608 on PI3Kα lipid kinase activity

S608 in the p85 subunit is a substrate for the protein kinase activity of PI3Kα [17, 20]. Phosphorylation of S608 by p110 is reported to down-regulate PI3Kα lipid kinase activity [17,20]. This raises the possibility that higher lipid kinase activities of oncogenic mutants of PI3Kα could be partly due to alterations in the levels of phosphorylation of S608.

To better compare lipid kinase activities of wild-type and mutant PI3Kα that were phosphorylated or unphosphorylated at S608, we opted to manipulate phosphorylation levels of recombinant p110αEE/p85α complexes *in vitro* rather than to make point mutations in S608. To perform lipid kinase assays with dephosphorylated PI3Kα, it was necessary to find a phosphatase that dephosphorylated p85α but not inositol lipids. We

compared three different protein phosphatases (Figure 2A). We have previously used Antarctic Phosphatase to dephosphorylate affinity-precipitated proteins for characterisation on 2D gels [21], however Antarctic Phosphatase was inactive at temperatures >4°C that are required for significant lipid and protein kinase activity of PI3Kα, and so had no effect on levels of phosphorylation of p85α or PI at room temperature (Figure 2A). Alkaline phosphatase is a broad spectrum phosphatase that was able to dephosphorylate both S608 and PI-3-P. In contrast, recombinant λ phosphatase dephosphorylated S608 but not PI-3-P (Figure 2A). λ phosphatase was therefore added to PI kinase assays to continually remove phosphate groups from S608 and allow comparison of the lipid kinase activity of phosphorylated and dephosphorylated PI3Kα.

As expected, inclusion of λ phosphatase in kinase assays resulted in no significant incorporation of ³²P-labelled phosphate groups into p85α, demonstrating that levels of p85α phosphorylation were low in the presence of λ phosphatase (Figure 2B, upper panel). Dephosphorylated p110αEE^{WT}/p85α did not have a significantly different PI kinase activity to p110αEE^{WT}/p85α that became phosphorylated on p85α over the course of the assay (Figure 2B, lower panel). Similarly, the E545K and H1047R mutants did not have significantly different PI kinase activities when p85α was either dephosphorylated or phosphorylated, although the PI kinase activities of p110αEE^{E545K}/p85α and p110αEE^{H1047R}/p85α were higher than that of p110αEE^{WT}/p85α as expected (Figure 2B, lower panel). Interestingly, as observed previously [15], the protein kinase activities of p110αEE^{E545K}/p85α and p110αEE^{H1047R}/p85α were not significantly different to that of p110αEE^{WT}/p85α (Figure 2B, upper panel).

It is possible that the stoichiometry of phosphorylation of S608 remains low after the 40 minute kinase reaction used, and thus lipid kinase activities of dephosphorylated and only partially phosphorylated p110αEE/p85α were compared in Figure 2B. We therefore performed a time-course to determine whether a difference in activity of phosphorylated and dephosphorylated p110αEE/p85α became apparent with increasing levels of phosphorylation of p85α. Levels of phosphorylation of S608 became saturated after approximately 30 minutes (Figure 2C, upper panel). In some assays, the level of phosphorylation of the E545K mutant was apparently higher than wild-type PI3Kα or the H1047R mutant, although this was an inconsistent observation and was not significantly different in most assays. When λ phosphatase was added, lack of incorporation of ³²P into p85α demonstrated that λ phosphatase continued to dephosphorylate S608 throughout the assay. (Figure 2C, lower panel, closed symbols). Despite apparently saturating phosphorylation of S608 between 40 and 120 minutes, the difference in the PI kinase activity of phosphorylated

Table 1 Kinetic analysis of *in vitro* lipid kinase activity of p110αEE^{WT}/p85α, p110αEE^{E545K}/p85α and p110αEE^{H1047R}/p85α

		Wild type	E545K	H1047R
PI kinase activity ^a	V_{max}^b	35 ± 13 ^c	118 ± 12	150 ± 13
	K_m (μM)	23 ± 3	22 ± 6	14 ± 7
PI-(4,5)-P ₂ kinase activity	V_{max}	35 ± 8	117 ± 13	146 ± 15
	K_m (μM)	22 ± 5	19 ± 6	9 ± 3

^aPI or PI-(4,5)-P₂ were used as substrates to generate PI-3-P or PI-(3,4,5)-P₃.

^bMichaelis-Menten kinetic parameters (substrate affinity (K_m) and maximum reaction velocity (V_{max})) for the production of PI-3-P or PI-(3,4,5)-P₃ were calculated from assays in which the concentration of ATP was varied.

^c K_m and V_{max} are shown as mean ± SEM, n = 4.

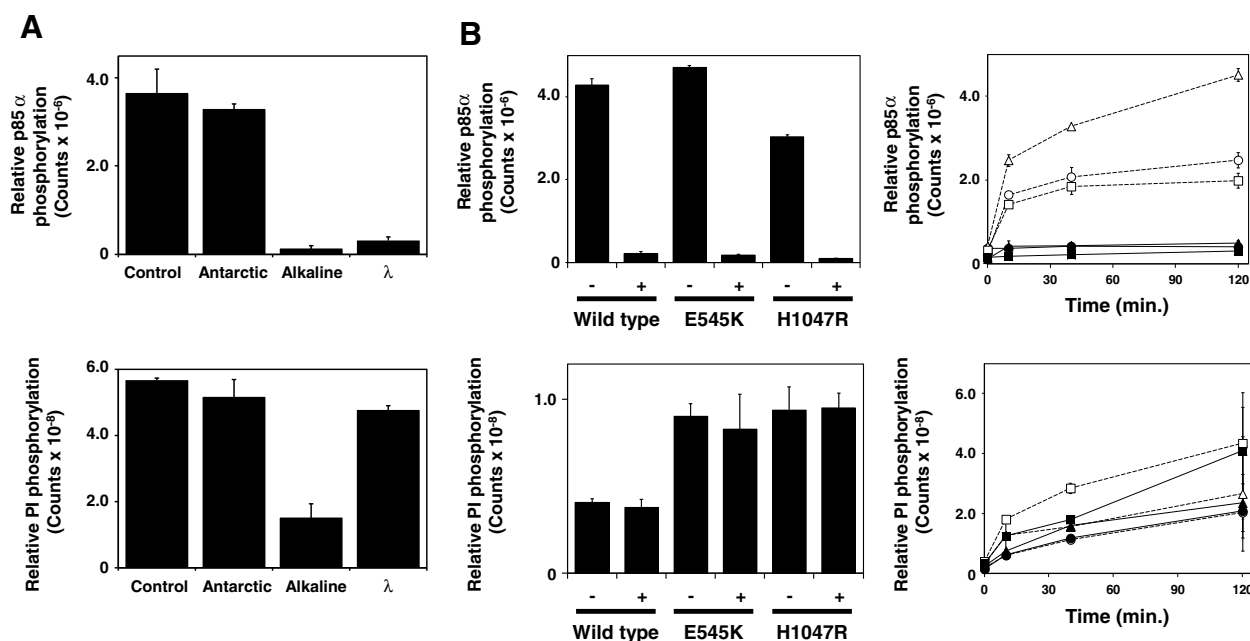


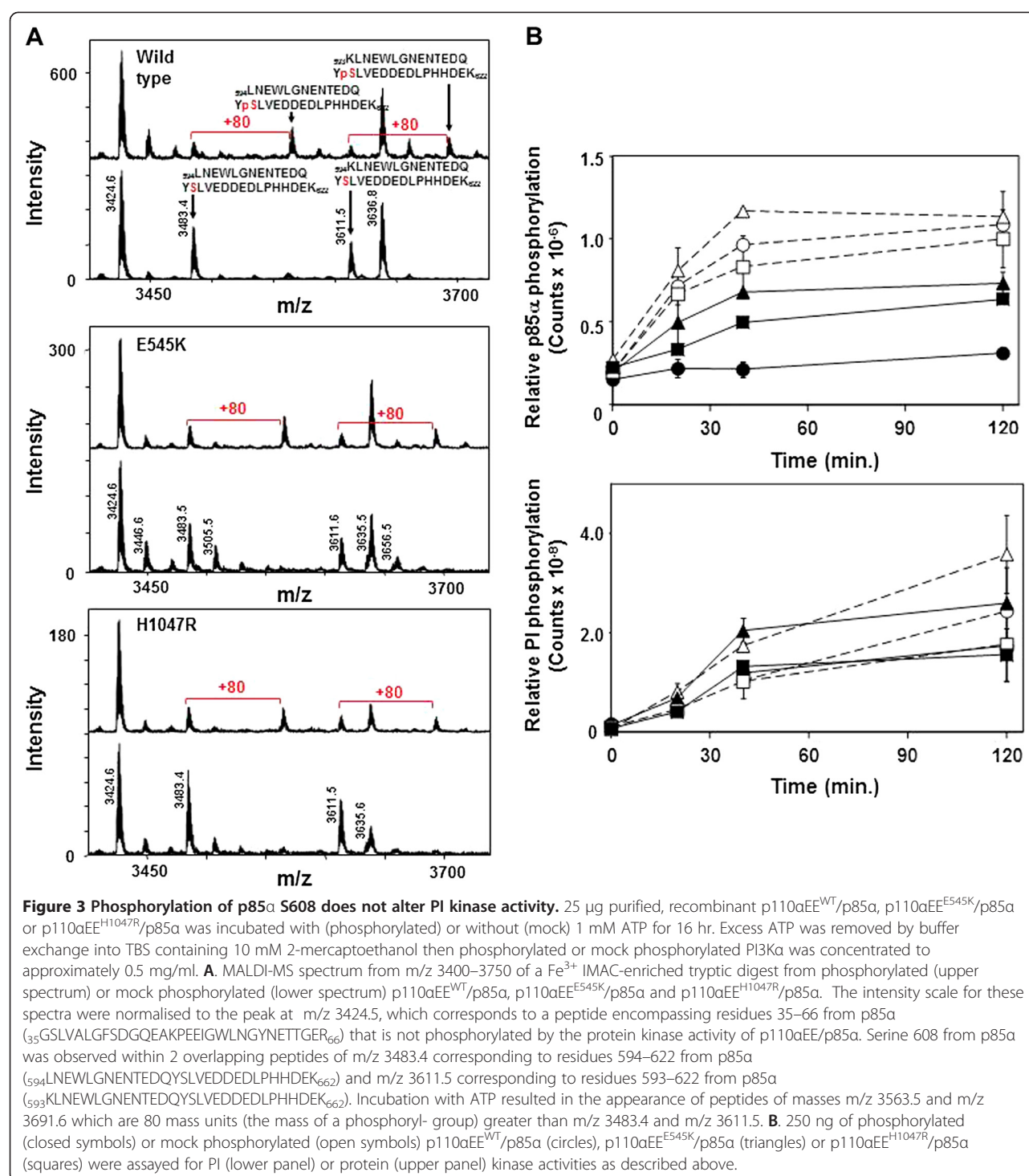
Figure 2 Dephosphorylation of p85 α S608 does not alter PI kinase activity. **A.** Dephosphorylation of PI3K α using different protein phosphatases. Purified, recombinant p110 α EE^{WT}/p85 α (200 ng) was assayed for protein (upper panel) or PI (lower panel) kinase activity in the presence of no phosphatase (control), 2 U Antarctic Phosphatase, 10 U Calf Intestinal Alkaline Phosphatase or 40 U Lambda (λ) Protein Phosphatase. Reactions were stopped after 30 minutes using 1 M HCl. **B.** Lipid kinase activity of dephosphorylated wild-type and mutant PI3K α . 200 ng purified, recombinant p110 α EE^{WT}/p85 α , p110 α EE^{E545K}/p85 α or p110 α EE^{H1047R}/p85 α was assayed for protein kinase activity (upper panel) or lipid kinase activity using PI as a substrate (lower panel) in the presence or absence of 40 U λ Protein Phosphatase. Reactions were stopped after 40 minutes using 1 M HCl. **C.** Time courses of phosphorylation of p85 α S608 and PI in the presence or absence of a protein phosphatase. 200 ng purified, recombinant p110 α EE^{WT}/p85 α (circles), p110 α EE^{E545K}/p85 α (triangles) or p110 α EE^{H1047R}/p85 α (squares) was assayed for protein kinase activity (upper panel) or lipid kinase activity using PI as a substrate (lower panel) in the presence (closed symbols) or absence (open symbols) of 20 U λ Protein Phosphatase. Reactions were stopped after the indicated times using 1 M HCl.

and dephosphorylated wild-type, E545K or H1047R PI3K α did not increase over time (Figure 2C, lower panel), suggesting that phosphorylation of S608 does not regulate the PI kinase activity in either wild-type or mutant PI3K α .

Effect of phosphorylation of p85 α S608 on PI3K α lipid kinase activity

To ensure that the apparent lack of difference in PI kinase activity of phosphorylated and dephosphorylated PI3K α was not due to a low stoichiometry of phosphorylation, even when levels were apparently saturated, we allowed the phosphorylation reaction to proceed for 16–24 hours using unlabelled ATP or no ATP for the mock phosphorylation reaction. Phosphorylated and mock-phosphorylated PI3K α were buffer exchanged to remove excess cold ATP, then the extent of phosphorylation of S608 was measured using MALDI-MS of tryptic digests of wild-type and mutant PI3K α . Overlapping tryptic peptides containing S608 were observed at m/z 3483.4 and m/z 3611.5 in mock or unphosphorylated PI3K α (Figure 3A, lower spectrum in each panel). Phosphorylation of PI3K α led to the appearance of peptides

at m/z 3563.5 and m/z 3691.6 (Figure 3A, upper spectrum in each panel), which are 80 mass units greater than the masses of the peptides containing non-phosphorylated S608 and suggest that these peptides incorporated one phosphate group per peptide. There was no evidence for phosphorylation of peptides other than those containing S608. To estimate the extent of phosphorylation of S608, we normalised the spectra to the height of a peptide at m/z 3426.6 that is not altered when PI3K α is phosphorylated, and compared the heights of the peaks of non-phosphorylated S608-containing peptides. Phosphorylation of p110 α EE^{WT}/p85 α led to almost complete disappearance of the peaks at m/z 3483.4 and m/z 3611.5 corresponding to non-phosphorylated S608, suggesting that at least 90% of p110 α EE^{WT}/p85 α was phosphorylated on S608 (Figure 3A, upper spectrum of panel 1). In support of this, the amount of ³²P incorporated into p85 α in a subsequent protein kinase time-course assay was negligible, suggesting that nearly all S608 residues were already occupied by an unlabelled phosphate group (Figure 3B, upper panel). The extent of phosphorylation of p85 α S608 in p110 α EE^{E545K}/p85 α and p110 α EE^{H1047R}/p85 α was less



than that observed for p110α^{WT}/p85α, as the heights of the peaks at m/z 3483.4 and m/z 3611.5 were less decreased by phosphorylation compared to the height of the same peaks in mock-phosphorylated p110α^{E545K}/p85α and p110α^{H1047R}/p85α (Figure 3A, panels 2 & 3), suggesting that a small proportion of mutant PI3Kα remained unphosphorylated after 16 hours. In agreement,

low levels of ³²P were incorporated into p85α in subsequent protein kinase time-course assays (Figure 3B, upper panel), suggesting that a small proportion of S608 in the E545K and H1047R mutants was not occupied by an unlabelled phosphate group. Nevertheless, the majority of p110α^{E545K}/p85α and p110α^{H1047R}/p85α was phosphorylated on S608 under these conditions.

The PI kinase activities of phosphorylated and mock-phosphorylated wild-type PI3K α were not significantly different over a 2 hour time-course. Similarly, the PI kinase activities of phosphorylated and mock-phosphorylated E545K and H1047R PI3K α were not significantly different, although the activities of H1047R, and to a lesser extent E545K, were higher than that of wild-type PI3K α . Nearly stoichiometric phosphorylation of S608 therefore did not affect the PI kinase activity of wild-type or mutant PI3K α , further suggesting that phosphorylation of S608 does not regulate PI kinase activity.

Discussion

As reported previously for bovine PI3K α [18], we have successfully expressed and purified recombinant complexes of full-length human p110 α and p85 α , the prototypic form of Class 1A PI3K using the strategy of selecting for the p110 α EE/p85 α complex by placing a 6 amino acid epitope tag at the C-terminus of the p110 α , the subunit with limiting expression levels. The addition of C-terminal EE tag to p110 α did not appear to affect PI3K α , as recombinant p110 α EE/p85 α had high stability and the expected activity and substrate specificity. High purity recombinant PI3K α allowed us to assess the relative phosphoinositide lipid and protein kinase activities of wild-type and two common, tumour-associated mutants of PI3K α .

Purified, recombinant oncogenic mutant forms of PI3K α had higher lipid kinase activities compared to wild-type PI3K α (Figure 1B) as expected [4-6,13]. The ATP K_m was not significantly different between wild-type and mutant forms of PI3K α (Table 1) as previously reported [19]. However, there was a significant difference in ATP V_{max} when either PI or PI-(4,5)-P₂ was used as a substrate, in agreement with a previous study [15]. This suggests that mutant forms of PI3K α phosphorylate and turn over inositol lipids more rapidly, rather than having inherent higher substrate affinities, thus generating increased levels of PIP₃ and potentially partially explaining increased downstream signalling when the PI3K pathway is activated.

PI3K α also has protein kinase activity and has been reported to phosphorylate a number of protein substrates [22,23] as well as its own p85 α regulatory subunit on S608 [16,17,20]. The role of S608 phosphorylation in the regulation of PI3K α lipid kinase activity remains controversial. Despite previous reports that phosphorylation of S608 down-regulates the lipid kinase activity of the p110 α catalytic subunit, we did not find any evidence to support this by *in vitro* manipulation of the levels of phosphorylation of S608 of purified, recombinant p110 α EE/p85 α using either a protein phosphatase or saturating pre-phosphorylation. The reason for this discrepancy is not clear. In two studies, semi-purified

recombinant PI3K α was used to show phosphorylation of S608 reduces the lipid kinase activity of p110 α [17,20]. Semi-purified PI3K α can be unstable, thus it is possible that the apparent decrease in the lipid kinase activity of semi-purified PI3K α over time was due to p110 α degradation rather than S608 phosphorylation. A number of other studies have not observed a decrease in lipid kinase activity of recombinant, purified or immunoprecipitated PI3K α in time-course assays [24-27].

The time-course of PIP₃ formation by endogenous PI3K in cells shows an initial increase, followed by a decrease in PIP₃ levels [28,29], but this is largely due to the action of inositol 3- and 5-phosphatases that directly dephosphorylate PIP₃ to form PI-(4,5)-P₂ or PI-(3,4)-P₂ respectively [30,31]. The phosphorylation of S608 in cells increases upon stimulation with insulin or PDGF [16], which mirrors activation of the lipid kinase activity of PI3K α , thus it is not clear whether phosphorylation of S608 down-regulates endogenous PI3K α activity. Mutation of S608 to a non-phosphorylatable residue has been shown to decrease lipid kinase activity rather than increase it, as would be expected if mutation to alanine prevented phospho-S608-induced down regulation of PI kinase activity [16], suggesting that mutation of this residue, which resides in the interSH2 domain of p85 α , affects PI3K α lipid kinase activity by altering the structure of PI3K α rather than by affecting the phosphorylation status.

Phosphorylation of S608 has also been reported to lead to dissociation of the p85 α and p110 α subunits [16]. Free p110 α has higher activity than p110 α in complex with p85 α but is very unstable and is quickly degraded [32], thus it is possible that the decrease in lipid kinase activity when p85 α S608 is phosphorylated could be explained by dissociation of p110 α and p85 α and rapid degradation of free p110 α . However, our highly purified recombinant forms of PI3K α , when phosphorylated to saturation on p85 α S608, showed no evidence of subunit dissociation by size-exclusion chromatography and no evidence of p110 α degradation by SDS-PAGE (data not shown). The lack of observed dissociation, degradation or decrease in lipid kinase activity concomitant with increasing p85 α S608 phosphorylation in our highly purified, recombinant PI3K α suggests that previous reports of S608 phosphorylation resulting in down-regulation of lipid kinase activity were due to the use semi-purified recombinant PI3K α which is non-specifically degraded over time.

A number of mechanisms that regulate PI3K α enzymatic activity have been described, but no single event has been demonstrated to result in full activation of this enzyme. It is likely that full activation of PI3K α is a multi-step process. Within the p110 α /p85 α complex, p110 α is both inhibited and structurally stabilised by tight binding to the p85 α subunit [32]. Activation of the

PI3K α involves protein-protein interactions that relieve the inhibition of the p110 α kinase activity that is due to inter-subunit interactions of p110 α with the N-terminal SH2 and inter-SH2 domains of p85 α [33]. One mechanism of activation of PI3K α is binding to specific phosphotyrosine-containing motifs (pYXXM) present in receptor tyrosine kinases (RTKs) and cytoplasmic signaling proteins, such as IRS-1, to the p85 α N- and C-terminal SH2 domains [34-36], which disrupts the inhibitory contact between the p85 α N-terminal SH2 and the p110 α catalytic domain [37,38]. The E545K mutation is thought to increase activity by disrupting the p85 α N-terminal SH2/p110 α interface, similar to binding of RTKs [37,38]. Binding of a range of other intracellular proteins, such as activated Ras, SH3 domain-containing proteins and small GTPases, have also been reported to activate PI3K α [39-41] but it is not clear whether binding of these ligands directly activates enzyme activity or whether binding results in activation by translocating PI3K α to the plasma membrane, where its lipid substrate is located [42,43]. Tyrosine phosphorylation of p85 α Y688 by Src family tyrosine kinases has also been shown to increase PI3K α activity [44], but the mechanism is not known.

Phosphorylation of p85 α S652 by PKC [45] (as well as p85 α S608 by p110 α) has been reported to decrease PI3K α activity. S608 is in the inter-SH2 domain of p85 α , but is not within the section that has been observed to contact p110 α [37,38,46], therefore it is not clear whether phosphorylation of S608 could influence the inter-subunit interactions of p110 α and p85 α . A model of the structure of p110 with the N-terminal SH2, C-terminal SH2 and inter-SH2 domains of p85 [47] suggests that the C-terminal part of the inter-SH2 domain (containing S608) and the C-terminal SH2 domain has the potential to contact the catalytic domain of p110. Activation of PI3K α due to binding of the p85 α C-terminal SH2 domain to phosphotyrosine-containing proteins would be expected to be due to a conformational change that disrupts the interface between the p85 α C-terminal SH2 domain and the p110 α catalytic domain, analogous to the disruption of the inhibitory contact between the p85 α N-terminal SH2 and the p110 α catalytic domain. In contrast, for phosphorylation of S608 to decrease PI3K α enzymatic activity, it would have to stabilise this interface and oppose the phosphotyrosine binding-induced disruption. Regulation of PI3K α by stabilisation of inter-subunit interactions has not yet been demonstrated.

Conclusions

Studies showing a decrease in PI kinase activity of wild-type PI3K α that correlated with increasing p85 α S608 phosphorylation suggested the possibility that decreased

S608 phosphorylation of oncogenic PI3K α could be a mechanism underlying its increased PI kinase activity. This study, in which highly purified, recombinant wild-type and oncogenic, mutant PI3K α was dephosphorylated or fully phosphorylated on serine 608 *in vitro* (thus avoiding point mutations which can potentially alter protein structure and activity), found no evidence that phosphorylation of S608 influenced PI kinase activity. In addition, levels of phosphorylation of oncogenic PI3K α were not different to that of wild-type PI3K α .

Methods

Production of recombinant proteins

cDNAs encoding full-length human p85 α (PIK3R1_HUMAN, [Uniprot: P27986], aa1-724) or full-length human wild-type or mutant (E545K or H1047R) p110 α (PIK3CA_HUMAN, [Uniprot: P42336], aa1-1068) with an additional C-terminal spacer (PGG) and a Glu- or EE-epitope tag (EFMPME) [18,48] were subcloned into the transfer plasmid pBlueBac4. Recombinant baculoviruses were produced by co-transfection of these transfer plasmids into Sf9 insect cells with Bac-N-Blue baculovirus DNA (Invitrogen), then plaque purified and amplified as described [49]. EE-epitope tagged p110 α (p110 α EE) and p85 α were co-expressed in exponentially growing Sf9 cells (density = $1.5-2 \times 10^6$ cells/ml) by infection with recombinant baculoviruses at a multiplicity of infection (MOI) of between 1 and 10. Wild-type or mutant p110 α EE/p85 α complexes were purified to homogeneity by anti-EE tag affinity chromatography and anion exchange chromatography as previously described [18,50]. The concentrations of purified, recombinant PI3Ks were quantified by UV spectroscopy using a molar extinction coefficient of $264115 \text{ M}^{-1} \text{ cm}^{-1}$.

Gel electrophoresis

Recombinant p110 α EE/p85 α complexes were separated by SDS-PAGE using 10% Tris-glycine gels. Fast Coomassie Blue staining and destaining was carried out using a microwave oven as described [51].

Phosphoinositide 3-kinase assays

PI3K assays were carried out essentially as previously described [18,52,53] in 20 mM Tris pH7.5, 150 mM NaCl (TBS) containing 5 mM 2-mercaptoethanol. PI kinase assays contained 2 mM MgCl₂, 2 mM MnCl₂, 0.2 mM ATP, 5–10 μCi [³²P] γ ATP, 500 $\mu\text{g/ml}$ of phosphatidylinositol (PI) and 250 $\mu\text{g/ml}$ of phosphatidylserine (PS). Extracted phospholipids were separated by thin layer chromatography in 65% 1-propanol, 0.7 M acetic acid, 50 mM phosphoric acid, exposed to a phosphor screen (Molecular Dynamics) and analysed using ImageQuant software (GE Healthcare). Michaelis-Menten kinetics for phosphorylation of PI and PI-(4,5)-P₂ were

calculated from initial reaction rates in assays in which the concentration of ATP was varied between 0 and 125 μ M. Reactions were stopped after 20 min using 1 M HCl.

Protein kinase assays

Protein kinase assays were carried out in TBS containing 2 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM ATP and 5–10 μ Ci [^{32}P]ATP. Phosphorylated proteins were separated from free [^{32}P]ATP by SDS-PAGE as described above. Gels were fixed and stained with Coomassie Blue, then dried, exposed to a phosphor screen and analysed as described above.

Phosphatase treatment

500 ng recombinant PI3K was mixed with 2–10 U recombinant Calf Intestinal Alkaline Phosphatase, 0.5–2 U Antarctic Phosphatase or 8–40 U Lambda (λ) Protein Phosphatase (NEB) and assayed for PI kinase activity or protein kinase activity as described above.

In vitro phosphorylation of recombinant p110 α EE/p85 α

25 μ g purified wild type or mutant p110 α EE/p85 α complex was incubated with or without 1 mM ATP in the presence of 20 mM Tris pH 7.5, 150 mM NaCl, 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$ and 2 mM $MnCl_2$ at room temperature for 16 hr. Phosphorylated or mock phosphorylated PI3K α was buffer exchanged into TBS containing 10 mM 2-mercaptoethanol using Sephadex G-25 (GE Life Sciences) and concentrated using centrifugal filters (Amicon Ultra15 10,000 NMWL, Millipore). 250 ng of phosphorylated or mock phosphorylated (control) PI3K α was assayed for PI kinase activity or protein kinase activity as described above.

Phosphorylation sites on p110 α EE/p85 α were mapped by digesting 2 μ g aliquots of phosphorylated or mock phosphorylated p110 α EE/p85 α with 0.1 μ g trypsin (Worthington) then purifying phosphopeptides using Fe^{3+} immobilised metal affinity chromatography (IMAC) as described [54]. Phosphopeptides were identified by peptide mass fingerprinting using a QSTAR oMALDI-QqTOF (Applied Biosystems/PE Sciex).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MJL designed the experiments; MJL, MS and NLC performed the experiments; MJL, MS, RBP and WAP analysed the data; MJL, WAP and RBP interpreted the data; MJL, WAP and RBP wrote the manuscript; MJL, WAP, RBP and CAM revised the manuscript; WAP and CAM gave final approval of the version to be published. All authors read and approved the final manuscript.

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