Comparison of the kinetic properties of the lipid- and protein-kinase activities of the p110α and p110β catalytic subunits of class-Ia phosphoinositide 3-kinases

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Growth factors regulate a wide range of cellular processes via activation of the class-Ia phosphoinositide 3-kinases (PI 3-kinases). We directly compared kinetic properties of lipid- and protein-kinase activities of the widely expressed p110α and p110β isoforms. The lipid-kinase activity did not display Michaelis–Menten kinetics but modelling the kinetic data demonstrated that p110α has a higher V_{max} and a 25-fold higher K_m for PtdIns than p110β. A similar situation occurs with PtdIns(4,5)P_2, because at low concentration of PtdIns(4,5)P_2 p110β is a better PtdIns(4,5)P_2 kinase than p110α, although this is reversed at high concentrations. These differences suggest different functional roles and we hypothesize that p110β functions better in areas of membranes containing low levels of substrate whereas p110α would work best in areas of high substrate density such as membrane lipid rafts. We also compared protein-kinase activities. We found that p110β phosphorylated p85 to a lower degree than did p110α. We used a novel peptide-based assay to compare the kinetics of the protein-kinase activities of p110α and p110β. These studies revealed that, like the lipid-kinase activity, the protein-kinase activity of p110α has a higher K_m (550 μM) than p110β (K_m 8 μM). Similarly, the relative V_{max} towards peptide substrate of p110α was three times higher than that of p110β. This implies differences in the rates of regulatory auto-phosphorylation *in vivo*, which are likely to mean differential regulation of the lipid-kinase activities of p110α and p110β *in vivo*.

Key words: p85α, PI 3-kinase.

INTRODUCTION

Phosphoinositides mediate a wide range of cellular functions and many effects of growth factors are thought to be mediated by transient alterations in the levels of the phosphoinositides phosphorylated on the D-3 position of the inositol headgroup [1,2]. In particular these are thought to be mediated by PtdIns(3,4,5)P_3, the levels of which are very low in quiescent cells but rise rapidly following growth-factor stimulation [3–5]. This rise is caused by growth-factor-induced activation of phosphoinositide 3-kinases (PI 3-kinases) in the cell. A number of PI 3-kinases have been identified, although only the class-I PI 3-kinases are capable of phosphorylating PtdIns(4,5)P_2 to produce PtdIns(3,4,5)P_3 [6,7].

Four genes encoding class-I PI 3-kinases have been identified. All encode proteins of about 110–120 kDa and all share a high degree of homology in the catalytic domain that is located towards their C-terminus. However, differences in structure outside the catalytic domain dictate the functional differences that divide these into class-Ia and class-Ib subclasses [6]. The class-Ib enzyme is attached to a 101-kDa regulatory subunit and is regulated by the binding of $\beta\gamma$ subunits of heterotrimeric G-proteins to both the catalytic and regulatory subunits [8]. The class-Ia PI 3-kinase catalytic subunit on the other hand binds to members of a highly homologous family of adapter subunits, which are distinct from the p101 class-Ib PI 3-kinase subunit and are all characterized by the presence of two SH2 domains (for a review, see [3]). Three isoforms of the class-Ia PI 3-kinase catalytic subunit have been identified, termed p110α [9], p110β [10] and p110δ [11], with the former two being widely expressed whereas expression of the latter is restricted to haematopoietic cells. Significant functional overlap between the isoforms might be expected as they share a high degree of sequence homology and, as far as has been determined, they bind to the same adapter subunits. However, there is growing evidence to suggest that p110α and p110β actually have quite distinct roles in the cell. Surprisingly, evidence is emerging that p110α and p110β have quite distinct subcellular localization, despite the fact that they associate equally well with the different adapter-subunit isoforms [12–14]. Further, there is growing evidence of functional differences, highlighted best by the finding that ablation of the *p110*α gene is developmentally lethal, indicating that p110β cannot fully substitute for p110α [15]. Functional differences have also been identified, with p110β but not p110α being activated by $\beta\gamma$ subunits [16], and autophosphorylation of p110δ down-regulates activity whereas no autophosphorylation of p110α was observed [17]. It is also clear that p110α is involved in cell growth and division as overexpression of the p110α catalytic subunit in cell-culture models increases mitogenesis [18], while neutralization of p110α blocks growth-factor-stimulated mitogenesis [19–21]. The other p110 isoforms can also play a role in mitogenesis in certain circumstances. While colony-stimulating factor-1-induced mitogenesis in macrophages [21] and platelet-derived growth factor (PDGF)-induced mitogenesis in fibroblasts [20] do not require activation of p110β, activation of this isoform is apparently required for stimulation of mitogenesis by insulin and lysophosphatidic acid [20]. There are also isoform-specific effects on other cellular functions. A recent report indicates that activation of p110δ, and to a lesser extent p110β, is required for migration of melanocytes, whereas activation of p110α is not required [21]. There is also growing evidence that p110α and p110β may play distinct roles in PI 3-kinase-dependent vesicle-trafficking events in the cell as p110β preferentially associates

Abbreviations used: PI 3-kinase, phosphoinositide 3-kinase; PDGF, platelet-derived growth factor.

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with rab5, a molecule that plays a key role in the cycling of endosomal vesicles [13]. Insulin also preferentially stimulates recruitment of p110β to a microsomal fraction that contains GLUT4 storage vesicles [12]. Evidence suggests that p110β is more responsive to insulin than p110α and, while overexpression of p110α stimulates glucose transport and GLUT4 translocation [18,22,23], that p110β is the most important isoform in this process [23]. Overall these studies clearly indicate that p110α and p110β have distinct roles in the cell. However, surprisingly little is currently known about the relative catalytic properties of either the lipid-kinase activities of p110α and p110β or the Mn^{2+}-dependent protein-kinase activity that these isoforms also possess [24,25]. In the current studies we have used recombinant forms of p85α/p110α and p85α/p110β to compare the kinetic properties of these two catalytic isoforms.

MATERIALS AND METHODS

Materials

Tissue-culture materials such as medium, PBS, trypsin and serum were obtained from Sigma. Also, chemicals were from Sigma unless otherwise stated. Monoclonal anti-Myc antibody (9E10) was generously given by Gerard Evan (ICRF, London, U.K.). Polyclonal anti-FLAG antibody was from Santa Cruz Biotechnology. Polyclonal anti-p85 antibody, raised against the N-terminal SH2 domain, was provided by Professor Kenneth Siddle (University of Cambridge, Cambridge, U.K.). [γ-^{32}P]ATP, [^{32}P]orthophosphate, [^{35}S]methionine and ^{125}I-Protein A were from Amersham. Phosphoinositides were obtained from Lipid Products, Redhill, Surrey, U.K. Bovine p85α and human p110α and p110β cDNAs and baculoviruses were provided by Professor M. D. Waterfield (Ludwig Institute, University College London, London, U.K.).

Expression of PI 3-kinase in HEK-293 cells

PI 3-kinase cDNAs were epitope-tagged using PCR. At the C-terminus of p85α was added a triple Myc tag encoding three iterations of the sequence (EQKLISEEDL) followed by a stop codon, and at the C-terminus of both p110 subunits (α and β) were added codons for a quadruple glycine linker, then the sequence DYKDDDDK (the FLAG epitope) followed by a stop codon. These were subcloned into pCDNA3.1 (Invitrogen). HEK-293 cells were transfected as described previously [26]. Expressed PI 3-kinase constructs were immunoprecipitated from supernatants using 9E10 anti-Myc epitope antibody and anti-mouse IgG–Sepharose (unless otherwise stated).

Expression of PI 3-kinase in Sf9 cells

Untagged p85α/p110α and p85α/p110β heterodimers were expressed from baculovirally transfected Sf9 cells as described previously [27]. Recombinant PI 3-kinase was purified using Actigel conjugated to a hexamer phosphotyrosyl peptide based on the sequence surrounding Tyr-751 of the PDGF receptor.

PI 3-kinase lipid-kinase assays

PI 3-kinase activity was measured in immunoprecipitates using two different methods. The reason for this was to rule out the possibility that any differences in kinetic properties were an artifact of the assay system.

Method 1 was based on a commonly used method [28]. PI 3-kinase activity was assayed in the pellets in buffer containing lipid in 3.2 mM $MgCl_2$, 20 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 30 mM NaCl, 1 mM dithiothreitol, pH 7.2, 0.2 % sodium cholate and 12 μM ATP containing 5 μCi of [γ-^{32}P]ATP. The reaction was run for 10 min at room temperature (unless otherwise stated) and stopped with 450 μl of chloroform/methanol (1:2, v/v). Lipids were extracted by adding 150 μl of chloroform and 150 μl of 0.1 M HCl to each tube and extracting the organic phase. Dried lipids were resuspended in 25 μl of chloroform/methanol/0.1 M HCl (200:100:1, by vol.) by vortexing. TLC plates were pretreated with (1 % potassium oxalate/2 mM EDTA)/methanol (1:1, v/v) and baked at 150 °C for 2 h. Lipids were separated on the TLC plates using methanol/chloroform/ammonia (concentrated)/H_2O (20:14:3:5, by vol.).

Method 2 was based on an alternative published method [29]. These assays were performed using the indicated concentration of lipid in 50 mM Tris/HCl (pH 7.4), 50 mM NaCl, 1 mM dithiothreitol, 3.2 mM $MgCl_2$ and 12 μM ATP containing 5 μCi of [γ-^{32}P]ATP. The reaction was run for 10 min at room temperature (unless otherwise stated) and stopped with 100 μl of 0.1 M HCl. Lipids were extracted by adding 200 μl of chloroform/methanol (1:1, v/v) then re-extracted by adding 80 μl of methanol/0.1 M HCl (1:1, v/v) to each tube and extracting the organic phase. Dried lipids were resuspended in 25 μl of chloroform/methanol/0.1 M HCl (200:100:1, by vol.) by vortexing. TLC plates were pretreated with (1 % potassium oxalate/2 mM EDTA)/methanol (1:1, v/v) and baked at 150 °C for 2 h. Lipids were separated on the TLC plates using propan-1-ol/2 M acetic acid (13:7, v/v).

In all cases assay results were quantified using a Fuji FLA2000 PhosphoImager. Kinetic analysis of lipid-kinase assays was performed using ARChekMAT software (ARC Scientific, Oxford, U.K.).

PI 3-kinase protein-kinase assay

In vitro phosphorylation of the adapter subunit of PI 3-kinase by the catalytic subunit was measured in immune complexes using a method based on that described previously by Dhand et al. [25]. Briefly, immunoprecipitates from transfected or control cells were washed three times in lysis buffer, twice in 500 mM LiCl/100 mM Tris/HCl, pH 8, and once in 1 % Tween 20 in PBS, and then they were transferred to peptide-assay buffer (5 mM $MgCl_2$, 50 mM NaCl, 5 mM $MnCl_2$, 20 mM Tris/HCl, pH 7.4, 0.1 mM sodium orthovanadate and 12 μM ATP with 5 μCi of [γ-^{32}P]ATP). The reaction was stopped by the addition of SDS/polyacrylamide gel loading buffer. The samples were electrophoresed on SDS/polyacrylamide gels, which were fixed and dried, and the extent of phosphorylation quantified by PhosphoImager.

Peptide phosphorylation assay

A peptide (RRLGNENTEDQYSLVEDDEDL) based on bovine p85α incorporating the p85α Ser-608 site (shown in bold) was used. The peptide was assayed as described for p85 phosphorylation above. The reaction was incubated at 25 °C and the rate of peptide phosphorylation was linear for up to 25 min. Incubations were stopped by the addition of polyacrylamide gel-loading dye. Samples were electrophoresed on 12.5 % Tris/Tricine SDS/polyacrylamide gels that were fixed and dried. Phosphorylation of the 2.5-kDa peptide band was quantified by PhosphoImager.

Phosphate labelling of HEK-293 cells

Wild-type or transfected confluent HEK-293 cells were washed twice in phosphate-free Dulbecco's modified Eagle's medium. Cells were then incubated for 2 h at 37 °C in phosphate-free

medium supplemented with 50 μCi/ml [^{32}P]orthophosphate. The p85/p110 complexes were isolated by immunoprecipitation with the 9E10 antibody as described above.

RESULTS

Expression of recombinant PI 3-kinase

Epitope-tagged p110α and p110β were co-expressed with Myc-tagged p85α in HEK-293 cells and levels of expression were determined by Western blotting with anti-FLAG and anti-Myc antibodies respectively (Figure 1). Transfection conditions were used which resulted in similar levels of p110α and p110β expression, as measured by anti-FLAG Western blot (Figure 1). The transient-expression protocol used resulted in expressed PI 3-kinase heterodimers that contained very little endogenous PI 3-kinase associated with the Myc-tagged p85α, as shown by the fact that when p85α was expressed alone no endogenous p110 became associated with the expressed adapter subunit (Figure 1; see also [26]).

To allow equal amounts of baculovirally expressed p110α and p110β to be used in assays the amount of purified recombinant PI 3-kinase expressed in Sf9 cells was determined by comparing the intensity level of the 110-kDa bands in Coomassie Brilliant Blue-stained SDS/PAGE gels.

Comparison of lipid-kinase activities of p110α and p110β

In initial *in vitro* lipid-kinase experiments using assay method 1 and PtdIns as a substrate the recombinant p110β produced in HEK-293 cells consistently displayed a lower lipid-kinase activity than p110α (Figure 2). Substantially similar results were obtained in similar assays using PtdIns(4,5)P_2 as a substrate. In these experiments p110β lipid-kinase activity towards PtdIns(4,5)P_2 (500 μM) was 43% that of p110α activity (±5%, S.E.M; n = 3). Therefore we performed a more detailed analysis of the kinetic properties of the lipid-kinase activities of these two enzymes. Both p110α and p110β expressed in HEK-293 cells display progress curves characterized by an initial lag in the formation of product (Figure 3) and thus cannot be analysed by

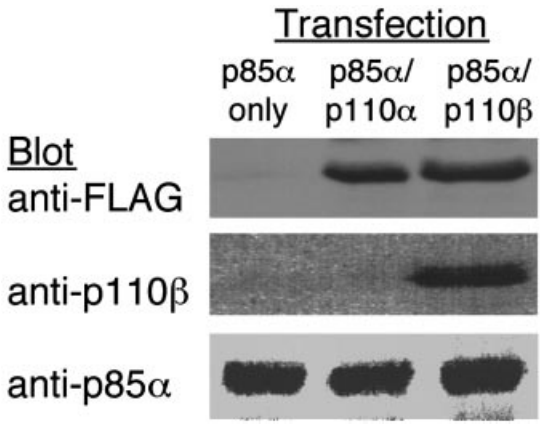

Figure 1 Transfection protocol results in similar amounts of p110α, p110β and p85α expression

Constructs containing Myc-tagged p85α or FLAG-tagged p110 isoforms were expressed in HEK-293 cells as described in the Materials and methods section. Cells were lysed and lysates were immunoprecipitated with 9E10 anti-Myc antibody then separated by SDS/PAGE and Western-blotted with the indicated antibody.

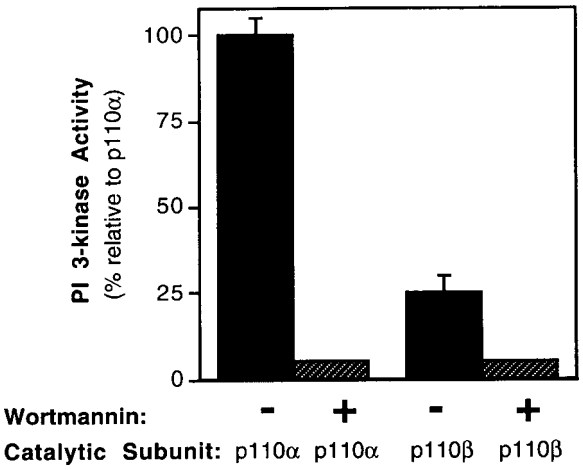

Figure 2 p110α displays a higher level of PI 3-kinase activity than p110β in standard lipid-kinase assay

p85α was co-expressed in HEK-293 cells with either p110α or p110β. The expressed complexes were immunoprecipitated via the Myc-epitope tag on the p85α, and PI 3-kinase assays were performed using PtdIns as a substrate as described in the Materials and methods section. Assays were performed in the presence or absence of 100 nM wortmannin as indicated. The amounts of p85α and p110α or p110β were determined by Western blotting for Myc and FLAG epitopes. The relative amounts of PI 3-kinase activity were normalized relative to the amount of p110α catalytic subunit present. Results show the means ± S.E.M. from three experiments.

the classical Michaelis–Menten model. However, curves could be fitted to this data using the ARChekMAT software utilizing methods we have described previously, which have been developed for analysis of non-Michaelis–Menten kinetics [30]. These results of the progress curves demonstrate clearly that at low lipid concentration p110β is more active than p110α while the reverse occurs at higher lipid concentration, indicating that p110α has higher K_m and V_{max} values than p110β (Figure 3). The best fit for the data over the range of PtdIns concentrations (6, 18, 56, 167, 500 and 1500 μM) at a constant ATP concentration (12 μM) was provided by a model which predicts the following. First, a primary phosphorylation takes place before the lipid is phosphorylated. Secondly, the overall equilibrium constant was assumed to be 1000 M^{-1} and the reaction was predicted to be highly exothermic. Finally, the absolute values of the kinetic parameters were difficult to obtain in these experiments because the number of data points per curve was only slightly greater than the number of unknown constants. However, the ratio of lipid K_m for p110α/p110β is a relative value, and so could be defined, and was 25. The ratio of V_{max}/K_m was well determined by the data and was 0.00383/s for p110α and 0.028/s for p110β. Despite the limitations in calculating an exact V_{max}, we found that our model still provided a good fit of the data using either of the two previously described K_m values for the lipid-kinase activity of baculovirus-produced recombinant p85α/p110α (i.e. 512 μM [31] or 130 μM [32]). The predicted K_m values of p110 isoforms expressed in mammalian cells in our studies fit with the previously published K_m value for class-Ia PI 3-kinase purified from liver. This had a K_m for lipid of 80 μM [33], which lies between the values our model would allow for p110α and p110β and so is consistent with our data, as the purified material from liver represents a mixture of p110α and p110β.

It is possible that the differences in kinetic properties may only arise due to the assay conditions used or result from the epitope-tagging method employed. Therefore we compared the lipid-

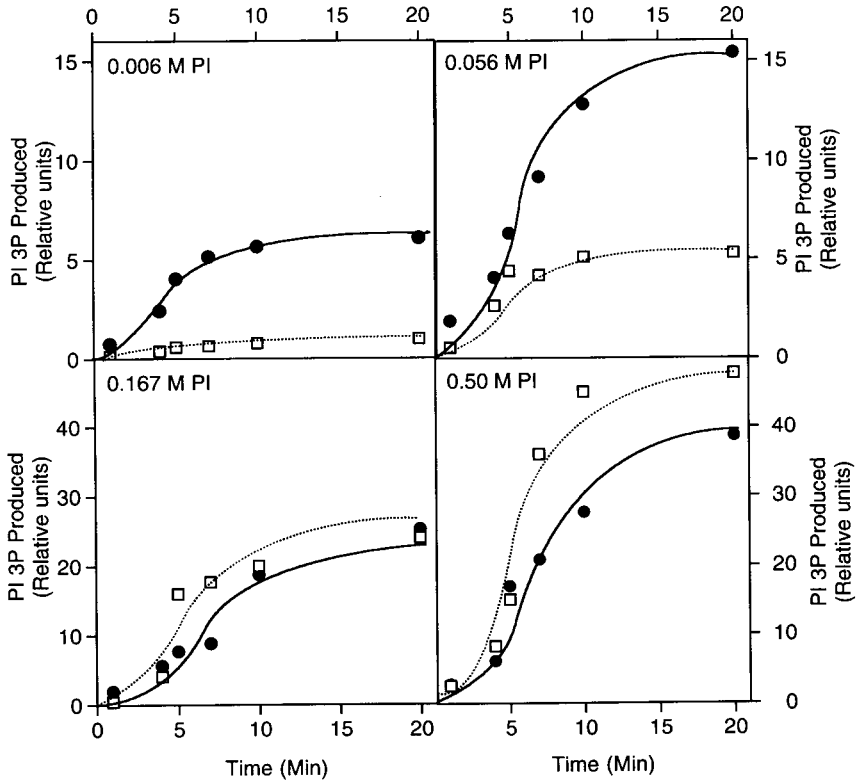

Figure 3 Kinetics of lipid kinase of recombinant PI 3-kinase

Myc-tagged p85α was co-expressed in HEK-293 cells with either FLAG-tagged p110α (□) or FLAG-tagged p110β (●). The expressed complexes were immunoprecipitated via the Myc-epitope tag on the p85α and PI 3-kinase assays were performed using PtdIns (PI) as a substrate using method 1 as described in the Materials and methods section. Reactions were stopped at the times indicated and the amount of lipid product formed was quantified. Results of typical experiments are shown. The progress curve for each reaction was that fitted by the ARChekMAT software. PI3P, PtdIns3*P*.

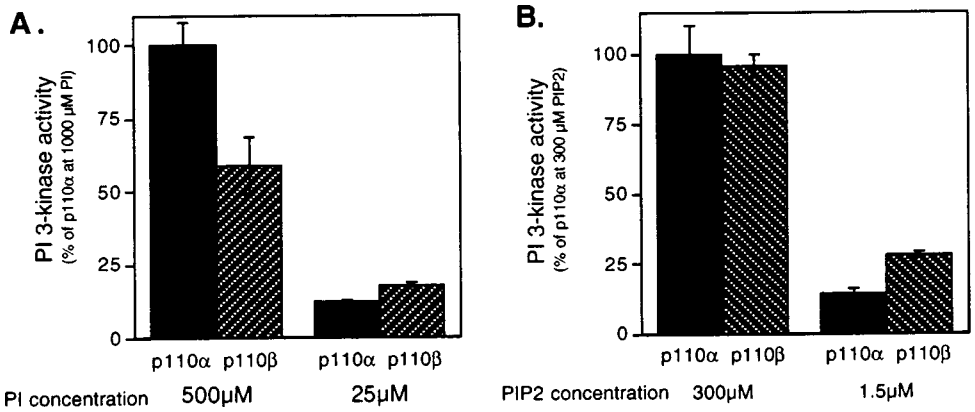

Figure 4 Comparison of PtdIns and PtdIns(4,5)P_2 kinase activities of recombinant p110α and p110β expressed in Sf9 cells

p85α was co-expressed in Sf9 cells with either p110α or p110β. The expressed complexes were purified using phosphotyrosyl peptide beads and PI 3-kinase assays were performed using PtdIns (PI) or PtdIns(4,5)P_2 (PIP2) as a substrate at the indicated concentrations using assay method 2 as described in the Materials and methods section. Similar amounts of p110α or p110β were used in each assay. Results show means ± S.E.M. from three determinations.

kinase activities of untagged p85α/p110α and p85α/p110β expressed in Sf9 cells (Figure 4). Importantly, at low substrate concentration p110β had a lipid-kinase activity that was more than double that of p110α, both towards PtdIns and PtdIns(4,5)P_2. However, this difference was reversed at high concentrations of substrate. This is consistent with p110α having a higher K_m for both PtdIns and PtdIns(4,5)P_2 and also suggests that p110α has a higher V_{max}, at least when PtdIns is used as a substrate. This also demonstrates that the differences in kinetic properties are not dependent on kinase-assay conditions as the PI 3-kinase assay method used for this second set of studies (Figure 4) differs greatly from that used in the first set of experiments (Figures 1–3), most notably in the absence of cholate in the latter assay system. The findings also argue that the epitope tagging was not

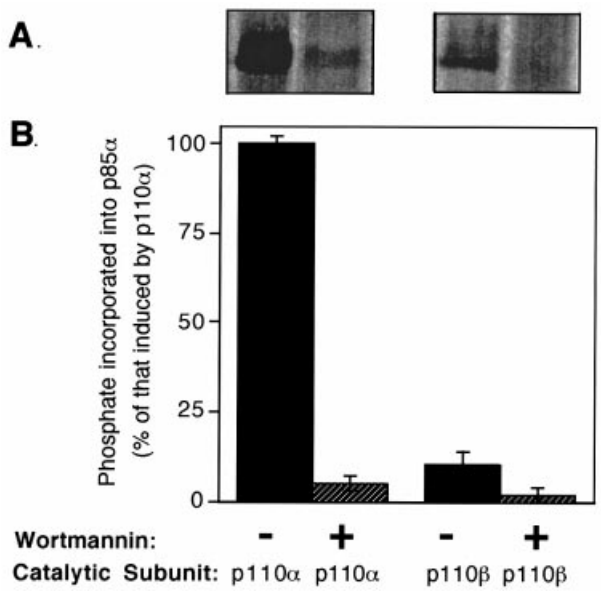

Figure 5 p110α displays a higher level of protein-kinase activity towards p85α than p110β

p85α was co-expressed in HEK-293 cells with either p110α or p110β. The expressed complexes were immunoprecipitated via the Myc-epitope tag on p85α and phosphorylation of p85α *in vitro* was determined as described in the Materials and methods section. Assays were performed in the presence or absence of 100 nM wortmannin as indicated. (**A**) Phosphorylated p85α was separated by SDS/PAGE and visualized and (**B**) phosphate incorporated into p85α was quantified using a Fuji FLA2000 PhosphoImager. The amounts of p85α and p110α or p110β were determined by Western blotting for Myc and FLAG epitopes. The relative amounts of PI 3-kinase activity were normalized relative to the amount of p110α catalytic subunit present. Results show the means $\pm$ S.E.M. from three experiments.

causing the kinetic differences. It is also worth noting that recombinant proteins expressed in the Sf9 cells were purified and assayed on phosphotyrosine beads that recruit p85 via the SH2 domain. Therefore the kinetic differences are apparently maintained even in the case where the SH2 domains are engaged by monophosphorylated peptides.

Comparison of serine kinase activity of p110α and p110β

It is known that in the presence of Mn^{2+} p110α can act as a protein kinase, causing phosphorylation of p85α at Ser-608, which in turn down-regulates the lipid-kinase activity of PI 3-kinase [24,25]. We have discovered recently that the same is true for p110β (P. R. Shepherd and C. A. Beeton, unpublished work). Therefore, if there are significant differences in the ability of p110α and p110β to phosphorylate p85 then this would cause a further difference in the *in vivo* lipid-kinase activity of these two isoforms and would add to the difference due to the kinetic properties of the lipid kinase. We therefore investigated the serine kinase activities of the p85α/p110α and p85α/p110β heterodimers expressed in HEK-293. *In vitro* kinase assays performed in these complexes revealed that there was no significant phosphorylation of either p110α or p110β but that there was significant phosphorylation of p85α in both cases, with this phosphorylation being inhibited by inclusion of 100 nM wortmannin in the incubation mixture, indicating that it was caused by the catalytic subunit (Figure 5). However, the *in vitro* kinase assays performed on the heterodimers revealed that there was a lower level of p85 phosphorylation in the heterodimers containing

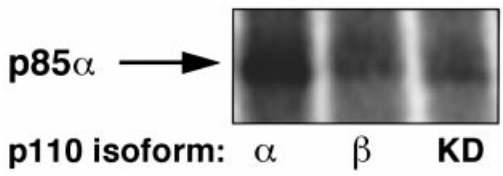

Figure 6 Comparison of abilities of p110α and p110β to phosphorylate p85α *in vivo*

p85α was co-expressed in HEK-293 cells with wild-type p110α (α), kinase-dead p110α (KD) or p110β (β). Cells were labelled with [^{32}P]orthophosphate as described in the Materials and methods section and the expressed complexes were immunoprecipitated via the Myc-epitope tag on p85α and separated by SDS/PAGE. Phosphorylated bands on dried gels were visualized using a Fuji FLA2000 PhosphoImager.

p110β (Figure 5). There are two alternative explanations for the lower overall lipid- and protein-kinase activities of p110β. First, this could represent a true difference in catalytic activity. Secondly, it could be that steady-state levels of Ser-608 phosphorylation are higher in p85α/p110β complexes than in p85α/p110α complexes. If this was the case then not only would there be fewer Ser-608 sites available for phosphorylation in the immunoprecipitated complex but the catalytic activity would also be reduced by the increased phosphorylation on Ser-608, and these together could explain the reduced *in vitro* phosphorylation of p85 by p110β.

To determine if the latter was the case, transiently transfected HEK-293 cells were labelled with ^{32}P and the p85α/p110α and p85α/p110β complexes were immunoprecipitated via the Myc tag on p85α. Conditions were used that resulted in similar levels of expression of p110α and p110β in the two different sets of transfections. These experiments first of all demonstrate that neither the p110α nor the p110β is phosphorylated *in vivo* when present in PI 3-kinase heterodimers. However, there is clearly phosphorylation of p85α *in vivo* although the level of p85α phosphorylation is much lower in the p85α/p110β heterodimers, where it is barely above the level seen in cells transfected with kinase-dead p110 (Figure 6). The finding that p85α is not phosphorylated in heterodimers containing p85α and kinase-dead p110 indicates that the phosphorylation of p85 is by the catalytic subunit. One implication of these findings is that the serine kinase activity of p110β towards p85α is actually less effective than that of p110α *in vivo*. This in turn suggests that the differences in lipid-kinase activity of p110α and p110β are indeed due to differences in the catalytic properties of the enzyme rather than to increased steady-state levels of phosphorylation of p85α by p110β. To resolve this issue in more detail we investigated the kinetics of the protein-kinase activity. It was not possible to analyse the kinetics of the phosphorylation of the Ser-608 site in p85α directly, as p110 and p85 are in a constant 1:1 ratio and so substrate concentration cannot be varied. Therefore we used a novel assay that we have developed recently which takes advantage of our finding that class-Ia PI 3-kinases can phosphorylate a peptide based on the Ser-608 region of p85α (P. R. Shepherd and C. A. Beeton, unpublished work). The velocity of the phosphorylation of this peptide was calculated at a range of different peptide concentrations (Figure 7). These studies revealed that the phosphorylation of the peptide displays Michaelis–Menten kinetics. Thus K_m for the Ser-608 peptide was calculated to be 550 μM for p110α and 8 μM for p110β and the ratio of relative V_{max} values of p110α/p110β was 3. Therefore serine kinase kinetics show a similar pattern to those of lipid kinase, with p110β having a lower K_m and lower V_{max}.

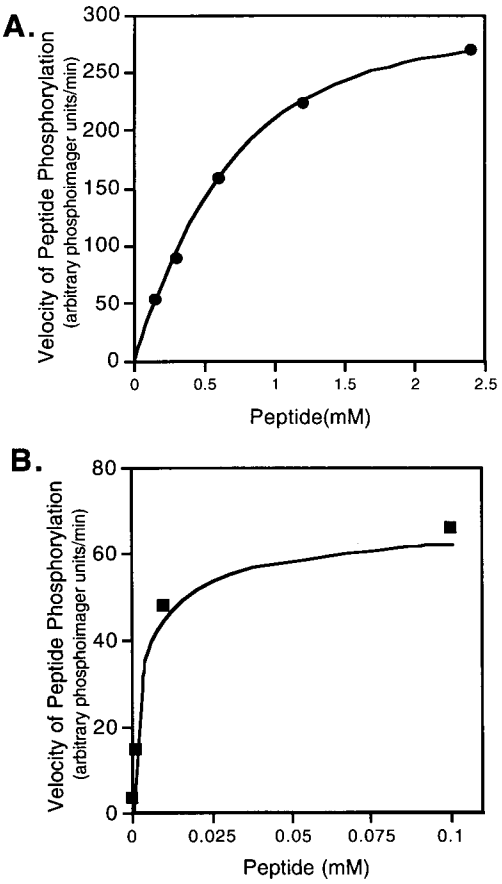

Figure 7 Kinetic characteristics of protein-kinase activity of p110α and p110β

p85α was co-expressed in HEK-293 cells with either p110α or p110β. The expressed complexes were immunoprecipitated via the Myc-epitope tag on p85α and phosphorylation of Ser-608 peptide was determined as described in the Materials and methods section. Time courses of Ser-608 peptide phosphorylation induced by either p85α/p110α (**A**) or p85α/p110β (**B**) were undertaken at a range of peptide concentrations and velocities of phosphorylation calculated and plotted against peptide concentration.

DISCUSSION

There are three highly homologous isoforms of the catalytic subunit of PI 3-kinase and it is increasingly apparent that whereas they have some overlap in function they each also have distinct roles in the cell [12,13,16,17,19,20,22,23]. It is therefore important to understand the basis of these differences. In many cases where different isoforms of a protein exist, differences in domain structure suggest differences in function. However, to date no major differences have been identified in the domain structures of p110α and p110β catalytic subunits of class-Ia PI 3-kinases. Neither has any difference been identified in the way these associate with the adapter subunits. Therefore we investigated the possibility that differences in kinetic properties of these isoforms may represent another mechanism by which they exert differential effects in the cell. The current study provides the first evidence that there are significant differences in the kinetic properties of p110α and p110β catalytic subunits of class-Ia PI 3-kinases.

The current studies demonstrate that p110α has a higher V_{max} and K_m than p110β for both PtdIns and PtdIns(4,5)P_2. This finding is consistent with the recent observation that expression of p110β in adipocytes caused a much smaller rise in the *in vivo* levels of D-3 phosphoinositides than did p110α [34]. Further differences in the catalytic properties of the two isoforms were identified by the finding that p110β is almost an order of magnitude more sensitive to wortmannin than p110α [34].

From an experimental point of view our findings have implications for the way growth-factor activation of PI 3-kinase activity is assayed. Generally PI 3-kinase lipid-kinase activity is assayed using methods very similar to that used in the current study. If assays were performed on immunoprecipitates where both p110α and p110β were present (e.g. anti-phosphotyrosine or anti-p85 immunoprecipitates) then the p110α would be contributing disproportionately to the observed PI 3-kinase activity at the concentrations of PtdIns generally used in these assays.

The most important potential implication of the kinetic differences between lipid-kinase activities of p110α and p110β lie at the functional level in the cell. Our assays were conducted *in vitro* using conditions that were unlikely to fully mimic the situation *in vivo*. However, if such kinetic differences are also found in the membrane environments present in real cells they would affect the location in the cell where these isoforms are most effective, as the concentration of the phosphoinositide substrates of PI 3-kinases varies in membranes. Of particular interest is the finding that localized concentrations of phosphoinositides are present in the low-density membrane lipid rafts [35–37]. Caveolae are invaginated forms of these membrane lipid rafts and also contain concentrations of a number of growth-factor receptors [38]. This suggests that p110α may work better than p110β in membrane caveolae and lipid rafts, with p110β being more effective than p110α in areas of the plasma membrane with lower concentrations of phosphoinositides. Little is known about the distribution of phosphoinositides in intracellular compartments, so it is unclear whether similar mechanisms might operate in the membranes of these organelles. It is known that different growth factors (e.g. insulin and PDGF) can activate spatially distinct pools of class-Ia PI 3-kinase [28,39]. There is also evidence that the isoforms of p110 are differentially regulated in these spatially distinct pools [12,13]. Therefore mechanisms such as those described above are likely to provide a mechanism by which such differences in the recruitment of PI 3-kinase isoforms to distinct intracellular locations can lead to specificity in growth-factor signal transduction.

We have also been able to analyse the kinetics of the Mn^{2+}-dependent protein-kinase activities of p110α and p110β using a novel peptide-based assay. These studies show that the kinetics of the protein-kinase activity of p110α and p110β follow a similar pattern to those of the lipid kinase, with p110α having a higher K_m and V_{max}. The K_m is unlikely to affect function in the cell as the p85 adapter subunit [24,25] and IRS-1 (insulin receptor substrate-1) [40,41] are the only confirmed protein substrates and both would be bound as 1:1 complexes with the catalytic subunit. However, the differences in protein-kinase V_{max} means that there may be differences in the steady-state levels of phosphorylation on p85. Indeed we find significant *in vivo* phosphorylation of p85α when complexed to p110α but not when complexed to p110β, which matches the result predicted from the kinetic properties. We have repeated the observation of other groups who demonstrated that phosphorylation of p85α by p110α causes a down-regulation of lipid-kinase activity [24,25], and we find too that p110β also phosphorylates p85α *in vitro* and causes down-regulation of the lipid-kinase activity (P. R. Shepherd and C. A. Beeton, unpublished work). Therefore our findings would suggest that in quiescent cells p110α activity is partially attenuated by phosphorylation on Ser-608 whereas this is not the case for p110β. It is currently not known if growth factors regulate the serine kinase activity of p110α and p110β but if this is the case

it would provide a novel mechanism for regulating PI 3-kinase activity.

In summary we find that p110α and p110β differ in the kinetic properties of both their lipid- and protein-kinase activities with p110α having a higher K_m and V_{max} for both activities. We conclude that the most likely consequence of this *in vivo* is that p110α and p110β will be maximally effective in different cellular locations and it provides further evidence that these two PI 3-kinase isoforms have distinct roles in the cell.

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