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Functional differences between two classes of oncogenic mutation in the PIK3CA gene

Claire Chaussade [a,c,1], Kitty Cho [a], Claire Mawson [b], Gordon W. Rewcastle [b,c], Peter R. Shepherd [a,c,*]

[a] Department of Molecular Medicine, University of Auckland, Private Bag 92019, Auckland, New Zealand
[b] Auckland Cancer Society Research Centre, University of Auckland, Private Bag 92019, Auckland, New Zealand
[c] Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Private Bag 92019, Auckland, New Zealand

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ABSTRACT

PIK3CA codes for the p110α isoform of class-IA PI 3-kinase and oncogenic mutations in the helical domain and kinase domain are common in several cancers. We studied the biochemical properties of a common helical domain mutant (E545K) and a common kinase domain mutant (H1047R). Both retain the ability to autophosphorylate Ser608 of p85α and are also inhibited by a range of PI 3-kinase inhibitors (Wortmannin, LY294002, PI-103 and PIK-75) to a similar extent as WT p110α. Both mutants display an increased V_{max} but while a PDGF derived diphosphotyrosylpeptide caused an increase in V_{max} for WT p85α/p110α it did not for the E545K variant and actually decreased V_{max} for the H1047R variant. Further, the E545K mutant was activated by H-Ras whereas the H1047R mutant was not. Together these results suggest helical domain mutants are in a state mimicking activation by growth factors whereas kinase domain mutants mimic the state activated by H-Ras.

PI 3-kinases phosphorylate the D-3 position on inositol head group of phosphoinoistides. There are three classes of PI 3-kinases, each with structural and functional differences [1,2]. All convert phosphophatidylinositol(4,5)bisphosphate into phosphatidylinositol(3,4,5)tris phosphate (PIP3), which in turn is able to bind to proteins containing PH domains. This results in the activation of a wide range of pathways that regulate diverse cellular functions including apoptosis, metabolism, migration and growth [2–4].

Oncogenic mutations of PIK3CA, which codes for the p110α isoform of class-IA PI 3-kinase, are relatively common in cancer, particularly in breast, ovarian and colon tumours [5,6]. These findings have led to the development and characterization of a wide range of small molecule inhibitors of PI 3-kinase with the aim of using these as cancer therapies [7–11].

To date no oncogenic mutations have been described in the other class-IA isoforms (p110β and p110δ), which raises the question of why only mutations p110α are oncogenic. A number of different mutations in p110α have been described and all those studied to date have been shown to increase PI 3-kinase signalling pathways and induce transformation when transfected into cells [12–17]. The great majority of mutations are found at two hotspot regions of the gene, one encoding the helical domain and the other coding for the kinase domain [18]. These are in very different regions of the p110α structure suggesting they may be acting in different ways [19–21]. This has been supported by recent functional evidence indicating that the two classes of mutation have different effects on cellular transformation [22].

The mutations could be affecting the function of PI 3-kinase by directly affecting the catalytic activity of the enzyme. However, they could also affect the way the activity of the enzyme is regulated by growth factors via the two SH2 domains in the p85α regulatory subunit. These engage with phosphorylated tyrosines in specific sequence motifs such as those found on many growth factor receptors or adaptor proteins causing conformational changes that allows an increase in catalytic activity of the p110α molecule [19,23,24]. There are at least two other mechanisms involved in the regulation of the class-IA PI 3-kinase complex. One of these involves the protein kinase activity of p110α which is capable of phosphorylating a range of substrates including H-Ras and the ser608 residue on the p85 subunit [25,26]. The latter phosphorylation causes inhibition of the enzyme complex [25,27,28]. As p110α is the only isoform of PI 3-kinase that possesses this activity [25,27] and also the only one that exhibits oncogenic mutations it is possible that the mutations could act by disrupting the autokinase activity. Finally, the activity of class-I PI 3-kinases is known to be regulated by Ras, both *in vitro* and *in vivo* [29–32]. Ras inter-

* Corresponding author. Address: Department of Molecular Medicine, University of Auckland, Private Bag 92019, Auckland, New Zealand.
E-mail address: peter.shepherd@auckland.ac.nz (P.R. Shepherd).
[1] Present address: Centre for Cell Signalling, Barts & The London School of Medicine, Queen Mary, University of London (QMUL), London.

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acts with a specific domain in p110 and so it is possible that the mutations in p110α could affect this interaction and so alter the regulation of the enzyme *in vivo* [21].

In this study we investigated the effect that the two major classes of *PIK3CA* mutation have on the biochemical properties of p110α. These studies identify significant biochemical differences between the two classes of mutant that provide an explanation for the functional effects observed *in vivo*.

Materials and methods

Materials. Unless otherwise stated other reagents were purchased from Sigma Chemicals, Auckland, NZ. PIK-75, PI-103 and polyclonal antibodies to p85 were obtained from Symansis (Auckland, NZ). Polyclonal antibodies to p110α, p110β and p110δ were kindly provided by Dr. Bart Vanhaesebroeck, Ludwig Institute, London. Polyclonal antibodies to phosphoser608 of p85α were as previously described [27]. Recombinant H-Ras (GV12) was purchased from Jena Bioscience, Jena, Germany. A doubly phosphorylated phosphotyrosyl peptide derived from the PDGF receptor (sequence CSDGGY(p)MDMSKDESVDY(p)VPMLD) was synthesised at the Department of Chemistry, University of Auckland.

Production of recombinant PI 3-kinase. The production of recombinant PI 3-kinase was as previously described [9]. The identity of the PI 3-kinases was verified by Western blotting and also by sensitivity to previously described isoform selective PI 3-kinase inhibitors.

Kinetic analysis and characterization of sensitivity to the PI 3-kinases inhibitors. IC_{50} values and kinetic constants were evaluated using a standard lipid kinase activity using phosphatidylinositol as a substrate basically as previously described [33]. The differences were; (a) that 100 μM cold ATP (or indicated) was used instead of 10 μM, (b) the DMSO concentration was 1% rather than 2% and (c) γ-^{33}P-ATP (GE Healthcare) was used instead of γ-^{32}P-ATP. The assays using phosphatidylcholine (PC) and phosphatidylserine (PS) used a molar ratio of PI:PS:PC of 1:1:1. The PDGF peptide was used at 10 μM in assays. The TLC plates were quantified using a phosphorimager screen (StormImager, Amersham). The reported IC_{50} values were determined by non-linear regression analysis (GraphPad Prism software). Both IC_{50} values and kinetic constants were determined on the basis of at least three independent experiments.

In vitro GTP loading of H-Ras. The loading of Ras with GTP−γ-S was performed by incubating purified recombinant protein (10 mM) in 20 mM EDTA in the presence of 2 mg mL^{-1} Bovine Serum Albumin (BSA) and 30 mM *n*-octylglucoside, and then mixed with 1 mM GTP−γ−S. The Ras was the incubated at 37 °C for 5 min with 20 mM $MgCl_2$ and the sample was put on ice.

Immunoblotting. Proteins were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corporation, USA). The membranes were incubated for 1 h in blocking buffer (20 mM Tris (pH 7.4), 137 mM NaCl, 0.5% (v/v) Tween 20) containing 3% (w/v) BSA (ICP Bio, Auckland, New Zealand) or non-fat milk and then incubated overnight in blocking buffer containing antibodies. Immunoreactive proteins were detected using horseradish peroxidase-linked secondary antibodies (Dako) and enhanced chemiluminescence (ECL) according to the manufacturer's instructions (GE Healthcare, Inc.). Signals were analyzed and quantified using Fuji LAS-3000 phosphorimager and Fuji Image Gauge software.

Statistical analysis. Results are presented as means ± SD with the number of experiments indicated in the legend. Statistical significance was assessed using the one-way ANOVA and Bonferroni's Multiple Comparison Test.

Results and discussion

Our first finding was that both the mutant forms of p110α retain the ability to autophosphorylate p85α on Serine 608 (Fig. 1). In wild type p85α/p110α this is an autoregulatory phosphorylation [25,27] but our results would indicate that the mutant forms of p110α are not as sensitive to this inhibitory phosphorylation.

We went on to investigate whether the mutations altered the inhibitory effects of five previously described PI 3-kinase inhibitors (Wortmannin, LY294002, PIK-75, PI-103 and SN 30693) [9,34,35]. We find that that there are only small differences in the IC_{50} of the drugs between WT p110α and the mutant forms (Table 1). The IC_{50}s were particularly close in the case of the three second generation PI 3-kinase inhibitors tested. Therefore we conclude that the oncogenic mutations in p110α do not confer any increased sensitivity or resistance to these drugs.

Previously there has only been a limited amount of information on the kinetic properties of the PI 3-kinase family [33,36–40] and only one report of the kinetic properties of the oncogenic forms of PI 3-kinase [40]. Our studies reveal that both classes of mutant show increased Km for ATP (Fig. 2A). This may be an artefact as the effect is not observed in an assay system containing PC and PS (Fig. 2A) or in enzyme stimulated with PDGF derived diphosphopeptide (Fig. 3A).

We also examined effects on V_{max}. Here we find that using the PI based PI 3-kinase assay that both the mutants have a significantly higher V_{max} than the wild type form of the enzyme. The use of a PC/PS based assay system lowers the V_{max} but both the E545K and the H1047R still show increased V_{max} in this assay system (Fig. 2B). Ef-

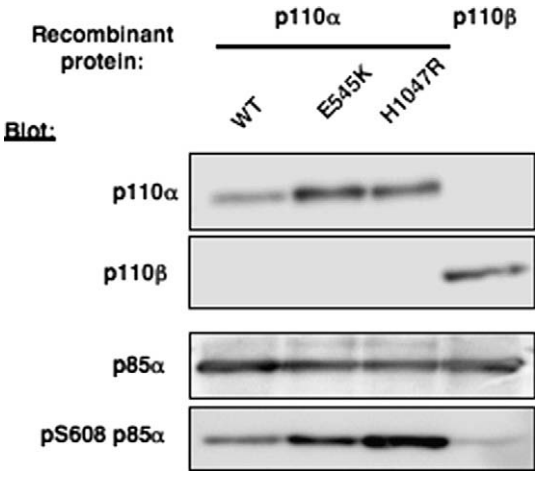

Fig. 1. Both mutant forms of p110α retain the ability to phosphorylate p85α on Serine 608. Recombinant p85α/p110α, either wild type or the two mutant forms (E545K and H1047R), were analyzed by Western blots using specific antibodies. Recombinant p85α/p110β was used as a negative control for the phosphorylation of p85α. Representative Western blots comparing the protein expression levels and phosphorylation levels are shown.

Table 1
IC_{50} values for selected PI3K inhibitors against lipid kinase activity. All IC_{50} values were determined using the PI 3-kinase lipid kinase assays using PI as a substrate as described in the materials and methods. Values are means ± SD. $n \geqslant 4$ for all determinations.

Compound	Most potent class I PI3K isoform targeted	p110		
		WT	E545K	H1047R
Wortmannin	α, β, δ	0.57 ± 0.07	0.78 ± 0.03	0.77 ± 0.05
LY294002	α, β, δ	500 ± 5	415 ± 4	715 ± 3
PIK-75	α	7.8 ± 1.7	7.2 ± 2.1	6.9 ± 1.9
PI-103	α	3.7 ± 0.5	4.4 ± 0.7	5.4 ± 1.3
PI540	α	231 ± 22	212 ± 38	199 ± 23