Supporting Online Material for

Mechanism of Two Classes of Cancer Mutations in the Phosphoinositide 3-Kinase Catalytic Subunit

Nabil Miled, Ying Yan, Wai-Ching Hon, Olga Perisic, Marketa Zvelebil, Yuval Inbar, Dina Schneidman-Duhovny, Haim J. Wolfson, Jonathan M. Backer,* Roger L. Williams*

*To whom correspondence should be addressed.
E-mail: rlw@mrc-lmb.cam.ac.uk (R.L.W.); backer@aecom.yu.edu (J.M.B.)

Published 13 July, Science 317, 239 (2007)
DOI: 10.1126/science.1135394

This PDF file includes:

Materials and Methods
Figs. S1 to S5
Table S1
References
Supporting Online Material

Materials and Methods

**Expression and purification of ABD/iSH2 complex.** The ABD of bovine p110α (residues 1-108) was PCR-amplified from a plasmid that was a gift from M. Waterfield and B. Vanhaesebroeck and the human p85α iSH2 domain (residues 431-600) was PCR-amplified from IMAGE clone 4290954 (this clone encodes variant 2 of p85α, which has a single amino acid difference (E451K) from variant 1; this residue is not conserved among p85-related subunits). Using an in-house polycistronic pOPCG vector, N-terminal GST-tagged ABD was co-expressed with non-tagged iSH2 domain in *E. coli* methionine auxotroph B834(DE3) in M9 minimal medium supplemented with amino acids, seleno-(L)-methionine and vitamins as described previously (1). Cells were grown at 37°C to OD\textsubscript{600} = 1.2 then induced with 0.3 mM IPTG and incubated overnight at 16°C. Cells were lysed with a French press, centrifuged in a Ti45 rotor (Beckman) for 30 min at 35,000 rpm and the filtered supernatant was incubated with Sepharose 4B glutathione resin (1.5 ml, GE Healthcare) at 4°C for 40 min. The resin was washed twice with 50 ml of buffer A (20 mM Tris-HCl pH 7.5 (25°C), 150 mM NaCl and 5 mM DTT). The protein was cleaved on the resin using tobacco etch protease (TEV) at a protease/sample mass ratio of 1/40 for 4 hours at 4°C. The supernatant was collected and the resin was washed with 10 ml of buffer A. The complex was passed through a 5 ml HiTrap heparin column (Amersham), washed with 80 ml of buffer A, and the flow-through and wash containing the complex was concentrated to 2 ml in a VivaSpin 5K concentrator (Sartorius). The concentrated sample was diluted 25-fold with buffer B (20 mM MES pH 6.5 (19°C), 60 mM NaCl and 5 mM DTT) and loaded onto a 5ml
Hi-Trap SP Sepharose column. The complex was eluted at approximately 170 mM NaCl with a gradient from 60 mM to 1 M NaCl in the same buffer. The complex was concentrated to 1 ml, loaded onto a 16/60 Superdex 75 gel filtration column (Amersham) and eluted at 4°C with gel filtration buffer (20 mM Tris pH 7.5 (25°C), 0.15 M NaCl, 5 mM DTT). The heterodimer was concentrated to 18 mg/ml, frozen in liquid nitrogen and stored at –80°C.

**Crystallization.** The initial crystallization conditions were obtained from a broad screen of 1440 conditions (2). Optimal selenomethionine-substituted crystals were obtained in hanging drops over reservoirs of 24-well VDX plates (Hampton) containing 0.7 ml of a solution consisting of 0.2M Mg(NO$_3$)$_2$, 20% PEG3350 (Hampton), 5mM Tris-HCl pH 7.0 (25°C), 5% glycerol and 5mM fresh DTT. The hanging drops contained 1 µl each of protein and reservoir solutions and 0.2 µl 45% PEG400 (Fluka). The crystals were cryo-protected by first adding 1 µl of a solution consisting of 35% PEG3350, 0.2M Mg(NO$_3$)$_2$ and 5mM Tris-HCl pH 7.0 (25°C) to the hanging drop, followed by transferring the crystals to 2 µl of the same solution for a few minutes at 4°C. Crystals were cryo-cooled by dunking in liquid nitrogen.

**X-ray diffraction, data collection, phasing and model refinement.** The complex crystallized in space group P2$_1$2$_1$2$_1$ with unit cell parameters $a=58.8$, $b=62.0$, and $c=74.7$. Multiple anomalous dispersion (MAD) data sets were collected at 100K on ESRF beamline ID14-4 using an ADSC CCD detector. Prior to data collection, a fluorescence spectrum for the crystal was obtained and two data sets were collected at wavelengths corresponding to the fluorescence peak (0.9793 Å) and a high energy remote (0.9393 Å). Images were integrated using MOSFLM (3) and scaled with SCALA (4). Table S1 lists statistics for data collection. Six putative Se sites were
located using the program SnB(5), and the substructure model was subsequently refined with autoSHARP (6). Solvent flattening was carried out with SOLOMON (7), using a solvent content of 36.1% as optimized by SHARP. An initial model was built using Arp/warp (8), and refined by alternating rounds of refinement with REFMAC5 (9), and manual rebuilding with the program O (10). Final statistics for the 2.4 Å resolution model are given in Table S1. A representative view of the electron density map is shown in Fig. S2. There are no residues in the disallowed regions of the Ramachandran plot and 96% of residues are in the most favored regions as defined by PROCHECK (11). Residues 1-15 and 106-108 of the ABD were not visible in the electron density. Electron density was visible for all residues in the iSH2.

The coiled-coil iSH2 that we see in the crystal structure is in close agreement with previous predictions (12) and with the EPR observations for the iSH2 in solution (13). Due to the limited set of spin-labeled sites, the EPR study did not identify the third helix \( \alpha_3 \). In light of the crystal structure, additional spin-labeled sites were employed and they confirm the presence of \( \alpha_3 \) in solution (Wu, H., Gerfen, G.J. and Backer, J.M., unpublished observations).

**Production of recombinant p110\( \alpha \) in HEK 293T cells.** Wild type and mutant N-myc-tagged bovine p110\( \alpha \), subcloned into the expression vector pSG5 (Stratagene), were expressed in HEK 293T cells grown in 10 cm plates by transient transfection. After two days, the cells were harvested by a quick wash in cold PBS, followed by resuspension in 1 ml lysis buffer (20 mM Tris pH 7.5, 137 mM NaCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\) containing 10% glycerol and 1% NP-40 (v/v), 100 \( \mu \)M sodium orthovanadate, and protease inhibitors (350 \( \mu \)g/ml PMSF, 100 \( \mu \)g/ml aprotinin, 5 \( \mu \)M leupeptin, and 1X Complete protease inhibitor cocktail (Roche)). After 20 min incubation at 4°C, with rotation, the lysate was clarified by
centrifugation, and then incubated with anti-myc antibody (5 µg, produced in JMB’s laboratory) overnight on a rotating wheel. Myc-p110α was immunoprecipitated by further incubation with 30 µl Protein G Sepharose beads for 1 h, and the beads were washed three times with PBS/1% NP-40, three times with 100 mM Tris pH 7.5, 500 mM LiCl₂, and two times with 10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA. Immunoprecipitates contained ~0.15 µg myc-p110α, estimated by resolving the immunoprecipitates on a coomassie-stained gel and quantifying the myc-p110α band using a LICOR Odyssey imaging device and a BSA standard curve (Fig. S3). Note that endogenous p85 in HEK 293T cells was not detectable in the immunoprecipitates (Fig. S3). The washed beads were resuspended in 60 µl of the final wash buffer, and were incubated without or with 100 µg purified p85ni for 4 h at 4°C with frequent agitation before PI3K assays (Fig. 3A).

**Production of recombinant p110α in Sf9 cells.** Cells grown in 35 mm dishes were infected with baculovirus encoding WT or E545K myc-p110α. After three days, the cells were resuspended in 500 µl of 10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA containing 350 µg/ml PMSF, 100 µg/ml aprotinin, and 5 µM leupeptin. The cells were lysed by three successive free-thaw cycles, in which the sample temperature was kept below 4°C at all times. The clarified lysates were assayed directly for PI 3-kinase activity since the specific activity of p110 expressed in Sf9 cells markedly decreases after immunoprecipitation (data not shown). However, PI3K activity in lysates bearing WT p110α was relatively stable over the incubation period of our assays (Fig. S4). PI3K activity in lysates of infected cells was generally 10-30 fold higher than in lysates of uninfected cells. Moreover, we observed similar inhibition of p110α whether p85ni was added directly to lysates, or to anti-myc immunoprecipitates (data
not shown). To compare inhibition of WT versus mutant p110α by p85ni, the amount of lysates was adjusted to have the same initial activity in all experiments, corresponding to ~0.7 nM myc-p110α in the assays (estimated by scanning western blot probed with anti-myc antibody, with quantified myc-p110α produced in HEK 293T cells as a standard). Lysates containing WT or E545K myc-p110α were incubated without or with p85ni (2.4 μM in Fig. 3B, 4.8-144 nM in Fig. 3C, and 14 μM in Fig. 3D), in a total volume of 60μl, either overnight (Fig. 3B) or for 6 h (Fig. 3C and D). Where indicated in Figure 3D, lysates were incubated for an additional hour in the presence of 100 μM phosphotyrosine-peptide derived from IRS-1 (DD(P)YMPMSPGAGAGAGAGAGNGD(P)YMPMspKS). PI3K activity in the mixtures was then measured directly.

**Expression and purification of p85ni.** The p85ni fragment of human p85α (residues 321-600) was subcloned into the vector pGEX-4T (Amersham Biosciences, Piscataway, NJ) and expressed as a GST fusion protein in *E. coli*. The purified protein was cleaved with thrombin and GST was removed by absorption on glutathione-Sepharose (Amersham Biosciences, Piscataway, NJ). The purified fragment was concentrated to 5 mg/ml and stored at -80°C. All point mutants of p110α and p85ni were produced with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by sequencing.

**PI3K assays.** The lipid kinase activity was assayed using phosphatidylinositol as a substrate as previously described (14, 15). Briefly, myc-p110α or mixtures of p110α/p85ni were incubated in the presence of 10 mM MgCl₂, 20 μg sonicated PI, and 100 μM ATP containing 10 μCi [32P]ATP (New England Nuclear NEG502A)
for 10 min with agitation at 22°C. The reaction was stopped with 20 μl 8N HCl, and the lipids were extracted with 160 μl CHCl₃:MeOH (1:1). A 50 μl aliquot of the lower phase was spotted on baked (1 h at 100°C) Whatman indicator-free aluminum-backed silica plates, which were developed in CHCl₃:MeOH:H₂O:NH₄OH (60:47:11.3:2), dried and analyzed using Phosphorimager cassettes (Molecular Dynamics).

**Modeling of a class of plausible models of the p110α/p85ni complex.**

There is no experimental structure for the p110/p85 complex, and this is an ongoing focus of our work. However, we wanted to explore whether we could better define an ensemble of plausible models for the p110/p85 heterodimer based on our structural and biochemical studies. As a start for this, the structure of the ATP bound crystal structure of p110γ (PDBid:1E8X(16)) was used to model the catalytic core of p110α. A study of the ABD/iSH2 interaction suggested that the ABD binds to the iSH2 as a rigid body with little or no conformational change in the iSH2 (17). Based on this assumption of rigidity, we searched for plausible ways to dock the iSH2/ABD unit on the catalytic core so that the models would conform to assumed distance constraints. The preliminary modelling assumed two distance constraints:

- In consideration of the autophosphorylation of the p85 Ser608 by the p110 catalytic subunit, the structure the ABD/iSH2 complex was docked onto the catalytic subunit so that p85α Ser608 would be in the vicinity of the p110α catalytic site. This assumes that the autophosphorylation of a p85 subunit is the result of catalysis by the catalytic subunit to which it is bound. Although this assumption has not been unequivocally established, it is the simplest explanation for the observation that the deletion variants of the p85 subunit can only be phosphorylated by p110 when they are bound in a complex with p110 via the iSH2 (18). Specifically, the distance between the CB of Asp964
in the catalytic site (a residue adjacent to the $\gamma$-phosphate of the enzyme-bound ATP) and the last ordered residue in the iSH2 structure, Asn600, was assumed to be less than 32 Å (i.e., we assumed that the eight residues between Asn600 and Ser608 might extend as far as $8 \times 4Å = 32 Å$).

- The distance between the C-terminal ordered residue in the ABD (Pro104) and the N-terminal residue of the p110 catalytic core (residue 144 in p110$\gamma$, corresponding to residue 108 in p110$\alpha$) is less than 16 Å (i.e., we assumed that the four residues between Pro104 and Arg108 might extend as far as $4 \times 4Å = 16 Å$).

Analysis with custom tailored versions of the CombDock(19) and PatchDock(20) algorithms that incorporated these two upper bounds on distances, suggested that the only way to accommodate the iSH2 as a rigid body in the context of the p110 catalytic core is to thread the iSH2 through a slot between the C2 domain and the kinase domain of the p110 subunit (Figure S5). These two loose distance constraints are not enough to uniquely determine a model, but they are sufficient to restrict the ensemble of possible models to a class that threads through the C2/kinase domain notch. We docked the nSH2 onto this p110$\alpha$/iSH2 model, taking into account the proximity of residues involved in inhibitory interaction between the helical domain and the nSH2 domain (Glu545 of p110$\alpha$, and Arg340 and Lys379 of p85$\alpha$ nSH2 domain). The connection between the nSH2 and the iSH2 domains was assumed to be flexible and only an upper-bound type distance constraint was applied. These additional distance constraints were:

- The C-terminus of nSH2 to N-terminus of iSH2: $<28 Å$, i.e., we assumed that the seven residues between the C-terminal end of the nSH2 domain and
the beginning of the first iSH2 helix might be flexible (4Å x 7 residues = 28Å).

- The distance between the K379 Cα and the E545 Cα: <13 Å, i.e., to take into account the nSH2/helical domain contact established by the charge-reversal experiment.

PatchDock modelling with these additional constraints established an ensemble of models. It should be noted that the constraints do not allow us to unambiguously define the orientation of the nSH2 with respect to the catalytic subunit. Our mutagenesis simply defines a K379/E545 contact about which the nSH2 could pivot to some extent.

We further manually built a model of this class so that the contact between the catalytic core and the iSH2 domain was rendered as intimately as steric hindrance allowed, as both the inner surface of the catalytic core slot and the corresponding surface on iSH2 are lined by conserved (from human to Drosophila), predominantly polar, residues. Supporting figure S5 illustrates our model of the p110/p85ni complex. Coordinates for this model can be downloaded from http://www.mrc-lmb.cam.ac.uk/rlw/text/p110p85model.html.
Supplemental Figures

**Figure S1.** Sequence alignments of ABD (A) and iSH2 (B) domains. Residues involved in heterodimer contact are labeled, symbols are color–matched to the secondary structure elements on which they reside (Figure 1B). Highly conserved surface residues in the ABD/iSH2 complex that are not part of the ABD/iSH2 interface are marked underneath the sequences. These may represent binding sites for other domains in the full-length p110/p85 complex (see Figure 2).
Figure S2. A representative view of the 2mlFol-DlFcl electron density map. A region of the interface between the coiled-coil iSH2 (red) and the ABD (yellow) is illustrated. The map is contoured at 1.2σ.
**Figure S3.** Myc-p110α produced in HEK 293T cells. (A). A coomassie-stained SDS gel resolving anti-myc immunoprecipitate from HEK 293T cells untransfected and transfected with myc-p110α vector. Immunoprecipitate of untransfected cells is devoid of endogenous p110, as well as lipid kinase activity. Endogenous p85 was also not observed in the immunoprecipitates of transfected cells. (B). Determination of specific lipid kinase activity of WT and mutants of myc-p110α as shown in Fig. 3A. The western blot (upper panel) was used for estimating the relative protein amounts, using a LICOR Odyssey imaging device. PI 3-kinase activity was measured using a Molecular Dynamics Phosphorimager.
Figure S4. PI3K activity in lysates of baculovirus-infected Sf9 cells. The lipid kinase activity in the lysates was relatively stable during the period between cell lysis and activity assay. The endogenous PI3K activity has been subtracted from all data.
Figure S5. A plausible p110/p85\textsubscript{ni} model. The ensemble of models that take into account the existing structures and satisfy the distance constraints all thread the iSH2 rod through a slot between the catalytic domain and the C2 domain. The model illustrated is representative of this ensemble.
### Supporting Table

**Table S1.** Data collection, structure determination and refinement statistics

#### MAD data collection and phasing statistics

<table>
<thead>
<tr>
<th>Data set</th>
<th>Peak&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Remote&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution</td>
<td>2.4 Å</td>
<td>2.4 Å</td>
</tr>
<tr>
<td>Completeness (last shell)</td>
<td>99.1 (100.0)</td>
<td>98.3 (100.0)</td>
</tr>
</tbody>
</table>
| $R_{\text{merge}}$
  (last shell) | 0.078 (0.42) | 0.048 (0.20) |
| Redundancy (last shell) | 7.0 (7.2) | 3.50 (3.6) |
| $<I/\sigma>$(last shell) | 14.3 (3.3) | 15.9 (2.4) |

#### Phasing statistics

<table>
<thead>
<tr>
<th></th>
<th>Peak&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Remote&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phasing power (iso)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA</td>
<td>0.8</td>
</tr>
<tr>
<td>Phasing power (anom)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Se sites found/expected</td>
<td>6/8</td>
<td></td>
</tr>
<tr>
<td>FOM after SHARP</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>FOM after SOLOMON</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>FOM after DM</td>
<td>0.88</td>
<td></td>
</tr>
</tbody>
</table>

#### Refinement statistics

<table>
<thead>
<tr>
<th></th>
<th>47.7-2.4 Å (10385, no cutoff)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Number of reflections)</td>
<td>2234</td>
</tr>
<tr>
<td>Protein atoms</td>
<td>17</td>
</tr>
<tr>
<td>Waters</td>
<td>0.23</td>
</tr>
</tbody>
</table>
| $R_{\text{cryst}}$
  (%) data used | 0.29 (4.8) |
| r.m.s.d. from ideality<sup>f</sup> | 0.013 Å/1.3°/5.4° |
| Average B (Wilson B factor) | 31 Å² (45) |
| RMSD B for bonded main (side) chain atoms | 0.7 (3.3) |

<sup>a</sup>$R_{\text{merge}} = \frac{\sum_{hkl} I_{0}(hkl) - \langle I(hkl)\rangle}{\sum_{hkl} I_{0}(hkl)}$.  
<sup>b</sup>The phasing power is defined as the ratio of the r.m.s. value of the heavy atom structure factor amplitudes to the r.m.s. value of the lack-of-closure error. 
<sup>c</sup>Data sets were collected at ESRF beamline ID14-4 at wavelengths 0.9794 Å and 0.9393 Å for the peak and remote data sets, respectively.  
<sup>d</sup>$R_{\text{cryst}}$ and $R_{\text{free}} = \frac{\sum ||F_{\text{obs}}|| - ||F_{\text{calc}}||}{\sum ||F_{\text{obs}}||}$; $R_{\text{free}}$ calculated with the percentage of the data shown in parentheses.  
<sup>e</sup>r.m.s. deviations for bond angles and lengths in regard to Engh and Huber parameters.
Supporting References