

Supplementary Materials for
**Regulation of 3-Phosphoinositide-Dependent Protein Kinase 1 Activity
by Homodimerization in Live Cells**

Thomas A. Masters, Véronique Calleja, Daven A. Armoogum, Richard J. Marsh,
Christopher J. Applebee, Michel Laguerre, Angus J. Bain,* Banafshé Larijani*

*To whom correspondence should be addressed. E-mail: banafshe.larijani@cancer.org.uk (B.L.);
a.bain@ucl.ac.uk (A.J.B.)

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Reference

Methods

Recombinant PDK1 activity assay

We monitored the incorporation of phosphate from ^{32}P -ATP into the PDK1 substrate PDKtide to determine the activities of recombinant GFP-PDK1, PDK1-Cherry, wild-type PDK1, and the isolated kinase domain (deleted PH domain) of PDK1. The reactions were performed at 30°C in a shaker. The recombinant PDK1 proteins were diluted in a dilution buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM dithiothreitol (DTT), and 100 $\mu\text{g/ml}$ BSA to stabilize the protein. PDKtide, the PDK1 substrate peptide, was purchased from Millipore and was provided in solution in distilled water at a concentration of 1.05 mM. The assays were performed in a final volume of 50 μl in the reaction buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM DTT, 10 mM MgCl_2 , 100 μM ATP (500 cpm/pmol), 100 μM PDKtide, and 100 ng of the recombinant PDK1 proteins. Incorporation of ^{32}P was stopped by spotting 10 μl of the reaction on P81 paper after 5, 10, and 20 min. The papers were washed 3 times in 30% acetic acid and counted in a liquid scintillation counter. Two independent control reactions were performed in which either the recombinant PDK1 or the PDKtide substrate were omitted in the reaction.

Purification of recombinant proteins

Purification of His-tagged recombinant proteins from frozen pellets was performed as follows. Pellets were thawed on ice and thoroughly resuspended in 50 ml of cell lysis buffer [50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, (pH 8.0), supplemented with 1% NP-40, 5 mM β -mercaptoethanol and 1 protease inhibitor tablet]. 4 ml of buffer was used per 1 to 2×10^7 cells for up to 2.5×10^8 cells per 50 ml of lysis buffer. Lysis proceeded for 10 minutes on ice. Cells were transferred to centrifuge tubes (Nalgene) and spun at 10,000g for 10 min at 4°C, with recombinant protein retained in the supernatant. Lysate supernatant was transferred to 500 μl of pre-equilibrated Ni-NTA agarose beads, and incubated on a rotary shaker for 2 hours at 4°C. To recover the beads, tubes were spun at 500g for 5 min at 4°C. Supernatant containing unbound protein was removed and saved for analysis. Beads were washed three times with 50 ml of wash buffer [50 mM NaH_2PO_4 , 300 mM NaCl, 15 mM imidazole, (pH 8.0)] containing a protease inhibitor tablet, and were recovered by centrifugation after each wash. Beads were transferred to a 1.5 ml Eppendorf centrifuge tube and spun at 450g at 4°C. After the beads were pelleted, all of the supernatant was removed and 0.5 ml of elution buffer [50 mM NaH_2PO_4 , 300 mM NaCl, 200 mM imidazole, (pH 8.0)] was added and the beads were gently resuspended and incubated at 4°C for 2 min. After centrifugation, the supernatant that contained diluted protein was recovered. The process was repeated a further three times to elute the maximum amount of protein from the beads. To remove the 6 \times His-tag from the recombinant proteins, 25 μl of tobacco etch virus (TEV) protease was added to each 0.5-ml fraction and incubated at 4°C overnight in an end-over-end rotor. Fractions were pooled and subjected to size exclusion chromatography (SEC) through a Superdex200 column (GE Healthcare) attached to an AKTA Explorer chromatography system (Amersham Pharmacia Biotech). Sample (2ml) was loaded onto the column and eluted in a buffer designed to maintain protein stability [50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM benzamidine, 1 mM EDTA, 5% glycerol] at a flow rate of 1 ml per minute. We monitored the absorbance of the fractions at 488 nm and 590 nm to detect PDK1 labeled with fluorescent protein, and 1.5-ml fractions were collected. Those fractions with substantial absorbance were subjected to SDS polyacrylamide gel

electrophoresis (SDS-PAGE) and Coomassie staining to check for purity. The fractions with the purest protein and the highest yield were pooled and protein was concentrated by repeated centrifugation in a VivaSpin-6 30 kD cut-off filter (Sartorius Stedim Biotech) to a total volume of not more than 1 ml. Protein concentration was determined by Bradford assay and each sample was aliquoted and stored at -80°C .

Determination of the total proportion of PDK1 dimers

FRET measurements yield the fraction of donor molecules that form complexes with acceptors. This is an underestimate of the true proportion of interacting PDK1 dimers and is represented by a factor dependent on the relative concentrations of donor and acceptor molecules. If the concentration of donors (GFP-PDK1) and acceptors (PDK1-mCherry) is given as $[GP]$ and $[PC]$ respectively, then the probability (p) that any particular PDK1 molecule in the cell is labelled with a donor molecule is given by,

$$p = \frac{[GP]}{[GP] + [PC] + [P_E]} \quad (\text{Eq. S1})$$

where $[P_E]$ is the concentration of endogenous PDK1. If $[P_E]$ is small with respect to $[GP]$, then the probability that any PDK1 molecule in the cell is bound to mCherry is approximately $(1-p)$. A PDK1 dimer can have one of several combinations of fluorescent protein labels, which occur with the probabilities shown in fig. S6. The proportion of dimers that possess both donor and acceptor molecules is $2p(1-p)$ and it is this fraction of the total population that is detected by FRET. In our experiments, the relative concentration of GFP-PDK1 to PDK1-mCherry was 1:2 (as determined by measurement of the total intensity from Hg lamp images of the cells). From (Eq. S1), p is thus $2/3$ and the proportion of dimers detected by FRET were $4/9$ of the total population (the maximum possible proportion is $1/2$ which occurs when the concentrations of donor and acceptor are equal). To obtain the true fraction of PDK1 dimers, the donor fraction detected by FRET was therefore multiplied by $9/4$. In figure 4 prior to stimulation the proportion of dimers detected by FRET was 0.1 and 5 min after stimulation the proportion was 0.18. These values correspond to a true interacting fraction of PDK1 dimers of 22.5% and 40.5%, respectively.

Pixel enrichment method

To analyze FRET in live cells, we used the method of pixel enrichment described by Calleja *et al.* (1) which works as follows. Increases in FRET are assessed by shifts in the lifetime histogram for the cell as it is imaged at a series of time intervals before and after stimulation. As the histogram shifts, it no longer overlaps with the lifetime histogram obtained before stimulation. Calculation of the degree to which the lifetime histogram no longer overlaps with that taken before stimulation reveals the enrichment at any particular time interval of short lifetime pixels (that is, the proportion of pixels that definitely have a lifetime shorter than the pixels in the first lifetime image). To implement this, lifetime histograms for all of the images from a particular cell in a time series (with T in min) were first normalized with respect to the area of the cell. To calculate the unmatched area, the initial histogram, $F_0(t)$, was subtracted from those at later times, $F_i(t)$, and the function describing the indefinite integral from $-\infty$ to each time-point, $f(t)$, was found. The peak value of this integral was taken as the proportion of enriched pixels. Thus for the i^{th} time point the enrichment η_i is given by:

$$\eta_i = \text{Max} \left\{ f(t) = \int_{-\infty}^t (F_i(t') - F_0(t')) dt' \right\} \quad (\text{Eq. S2})$$

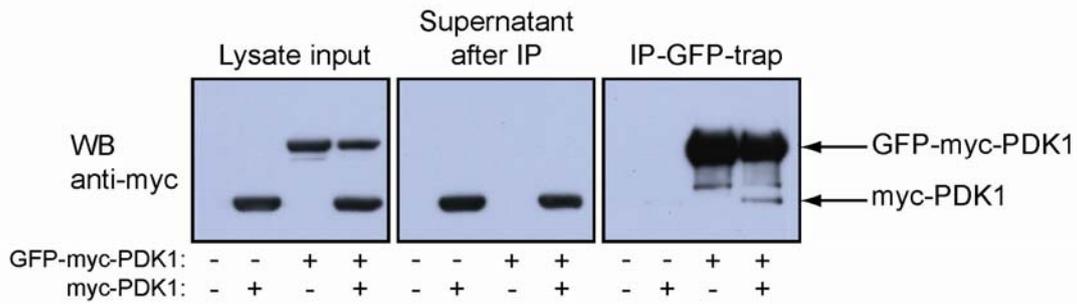


Fig. S1. Coimmunoprecipitation of myc-PDK1 with GFP-myc-PDK1 from COS-7 cells. The GFP-trap system was used to pull down GFP-myc-PDK1, and the proteins were detected by Western blotting analysis with an antibody against the myc tag. The left panel shows the relative abundances of myc-PDK1 and GFP-myc-PDK1 in each condition in the total cell lysate. The middle panel shows the myc-PDK1 proteins that remained in the supernatant after immunoprecipitation with GFP-trap. The right panel shows the coimmunoprecipitation of myc-PDK1 with GFP-myc-PDK1.

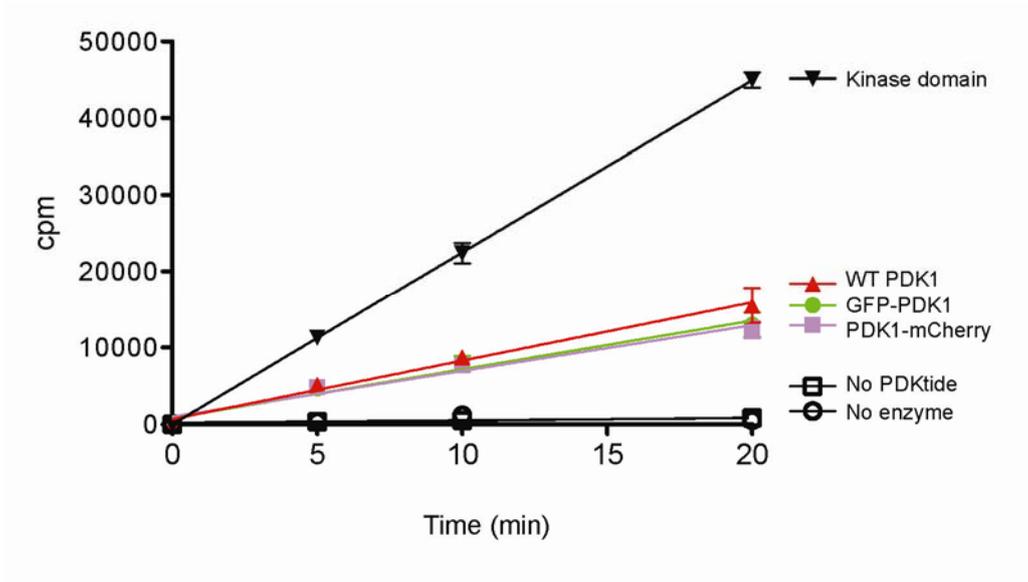


Fig. S2. In vitro phosphorylation of the PDKtide peptide by recombinant tagged PDK1. GFP-PDK1 (green); PDK1-mCherry (magenta); wild-type (WT) PDK1 (red); isolated PDK1 kinase domain (black triangles); negative control without PDK1 (open circles); negative control with GFP-PDK1 but without PDKtide substrate (open squares). The straight lines are fits to the data points. Data shown are the average of three independent experiments. The incorporation of ^{32}P from ^{32}P - γATP into PDKtide was measured at 5, 10, and 20 min. The data show that the addition of GFP or mCherry to PDK1 did not modify its activity. The isolated kinase domain of PDK1 exhibited an increased activity relative to that of full-length WT PDK1.

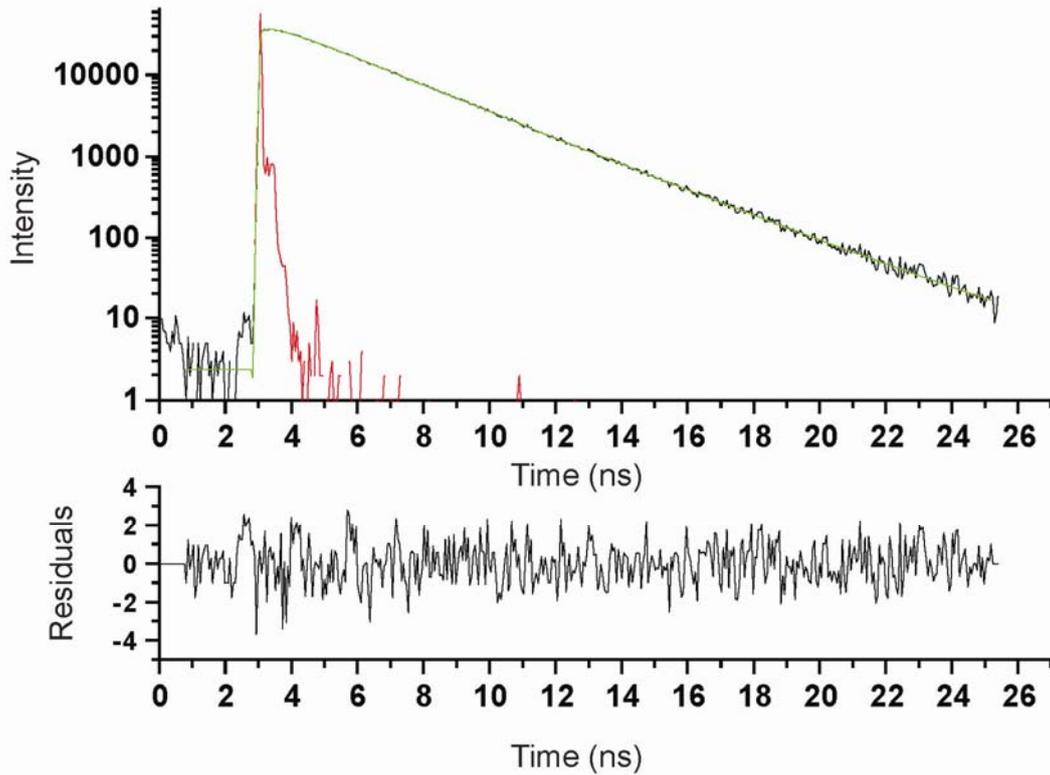


Fig. S3. Analysis of sensitized acceptor fluorescence. Deconvolution of the emission from a sensitized acceptor (mCherry) in conditions of equimolar GFP-PDK1 and PDK1-mCherry yields a multi-exponential fit that is characterized by a growth time of 0.69 ns and a bi-exponential decay (2.187 ns and 2.876 ns). Excitation conditions corresponded to an incident (on-sample) power of $18 \pm 0.5 \mu\text{W}$, giving a detection count rate of $1.0 \pm 0.1 \times 10^3$ cps. A similar result was obtained for GFP-PDK1-mCherry, whereas for PDK1-mCherry and the control sample of equimolar isolated GFP and mCherry, no rise in acceptor fluorescence was noted, indicating the absence of FRET in these systems (see Table 2 for the full results of these experiments).

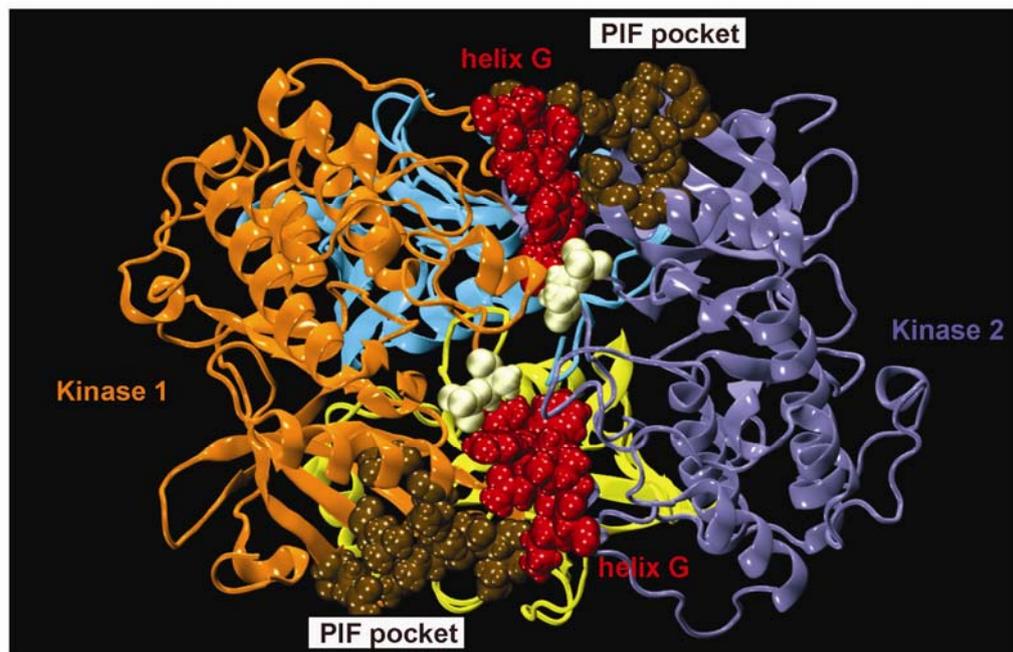
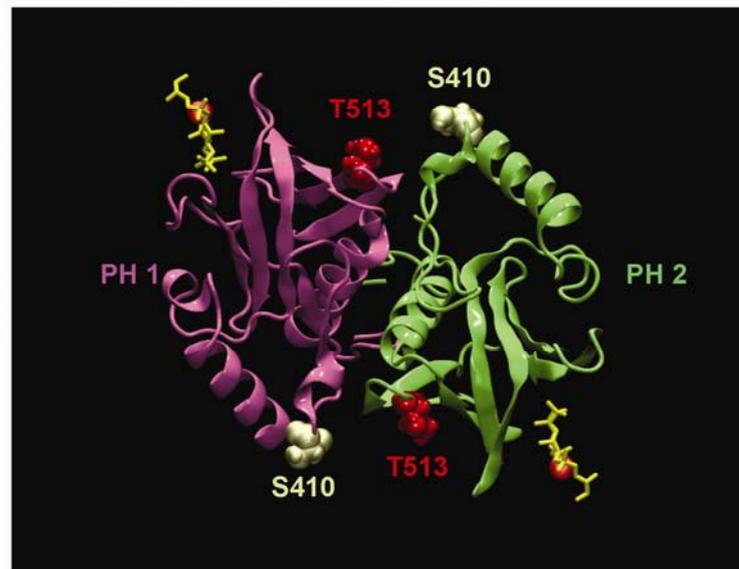
A**B**

Fig. S4. Molecular modeling of the PDK1 homodimer. **(A)** Interaction of the α G helix of one kinase (red) with the PIF pocket (brown) of the other. **(B)** Detailed view of the PH domain interaction showing the close proximity of Thr⁵¹³ (red) of the PH domain of one monomer to Ser⁴¹⁰ (gold) of the other.

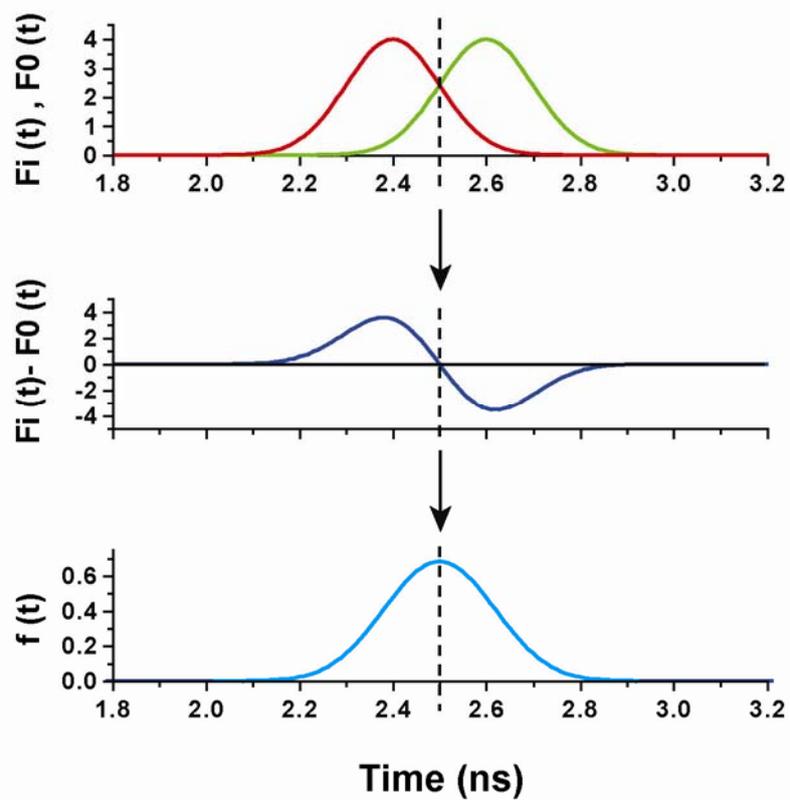


Fig. S5. Example of the calculation of pixel enrichment. The normalized lifetime distributions of two cells are represented by $F_0(t)$ in green and $F_i(t)$ in red. The difference in the functions $F_i(t) - F_0(t)$ is shown in purple. The function $f(t)$, which describes the indefinite integral from $-\infty$ to each time point, is in cyan.

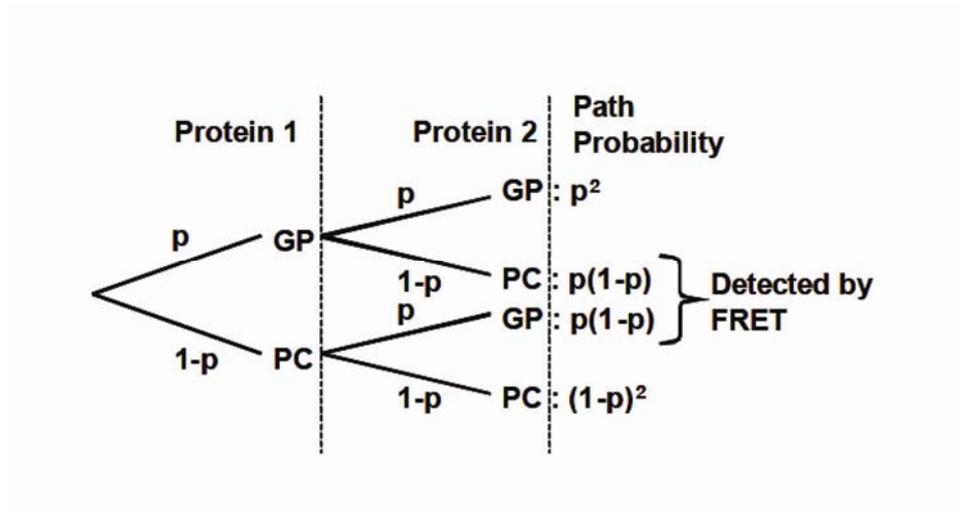


Fig. S6. Probabilities that a PDK1 dimer can be detected by hetero-FRET. By considering the two PDK1 molecules in a dimer as independent, it can be seen that observable FRET pairs only occur on the two central “paths” in probability space; two PDK1 dimers labelled with two GFP or two mCherry molecules have no effect on the observed donor lifetime. GFP-PDK1 (GP) and PDK1-mCherry (PC).

Reference

1. V. Calleja, D. Alcor, M. Laguerre, J. Park, B. Vojnovic, B. A. Hemmings, J. Downward, P. J. Parker, B. Larijani, Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo. *PLoS Biol.* **5**, e95 (2007).