**Unravelling the effect of the E545K mutation on PI3Kα kinase†**

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PI3Kα controls several cellular processes and its aberrant signalling is implicated in tumorigenesis. One of its hotspot mutations, E545K, increases PI3Kα lipid kinase activity, but its mode of action is only partially understood. Here, we perform biased and unbiased molecular dynamics simulations of PI3Kα and uncover, for the first time, the free energy landscape of the E545K PI3Kα mutant. We reveal the mechanism by which E545K leads to PI3Kα activation in atomic-level detail, which is considerably more complex than previously thought.

**Introduction**

Members of the phosphoinositide-3 kinase (PI3K) family control several cellular responses. PI3Kz, a class IA PI3K isoform, catalyses the phosphorylation of the inositol lipid PIP2 at the 3' position of the inositol ring, generating the signalling molecule PIP3, which controls cellular activities including proliferation, survival, and motility. Elevated PI3Kz signalling can contribute to oncogenesis and is a hallmark of human cancer.⁴

A number of structural and biophysical studies, motivated by the role of PI3Kz in cancer development, have shed light on its architecture and the regulation of its activity.² PI3Kz is comprised of a catalytic subunit, p110z, and a regulatory subunit, p85z (Fig. 1). The p110z subunit of PI3Kz associates with the p85z subunit, which contains two SH2 domains (nSH2, cSH2) flanking a region known as the inter-SH2 (iSH2) domain (Fig. 1). The catalytic and regulatory subunits form contacts that maintain the enzyme in a low activity state in the absence of a signal. Binding of phosphotyrosine (pY)-containing peptides to the nSH2 relieves these inhibitory contacts and positions the dimer near the plasma membrane, where it can access and phosphorylate the lipid substrate PIP2 in the presence of ATP as well as receive further inputs from Ras and other signalling components.²⁻⁵

PI3Kz is the most commonly mutated kinase in cancer, and PIK3z-activating mutations have been identified in human tumours, clustering in two hotspot mutations, H1047R and E545K.⁶ The hotspot mutation E545K in the helical domain of p110z has been found to increase PI3Kz lipid kinase activity.⁷ In this study, we unravel, for the first time, the conformational free energy landscape of E545K PI3Kz and elucidate the effect of the charge reversal mutation E545K on the dynamics and function of PI3Kz using the largest enhanced sampling calculations to

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**Fig. 1** PI3Kz domain organisation of the p110z (catalytic) and p85z (regulatory) subunits. The E545K (yellow van der Waals spheres) point mutation lies at the interface between the nSH2 (p85z) and helical (p110z) domains. Grey regions in p85z have not been simulated due to the absence of structural information.
Results and discussion

Unbiased MD simulations

In the unbiased MD simulations, Glu545 in the WT PI3Kζ forms salt-bridges with Lys379p85 and Lys548p110a, a backbone hydrogen bond with Leu380p85a, and participates in hydrophobic interactions with the side chain of Ile381p85a through the aliphatic part of its side chain (Fig. S1†). Lys379p85a is part of a highly conserved phosphopeptide binding surface on nSH2, and a charge reversal mutation of Lys379p85 (K379E) has been reported to induce increased lipid kinase activity, highlighting the importance of the interactions it participates in.19

The repulsive potential between the positively charged side chains of Lys545 and Lys379p85a in the E545K PI3Kζ is expected to trigger the disruption of the nSH2–helical contacts, including the backbone hydrogen bond between Lys545 and Leu380p85a, which was shown to be the last point of contact between the two domains in our previous work,12 and lead to the detachment of the nSH2 from the helical domain. Indeed, in previous work, we observed a spontaneous rotation of the nSH2 domain away from the helical domain driven by the E545K mutation,12 which did not have an immediate effect on the conformation of the kinase domain. Interestingly, in this work, in two out of the three independent unbiased simulations, we observe nSH2 moving away from the kinase and C2 domains (Fig. S2†), which drives the release of the iSH2 regulatory contacts from the activation loop, without actually detaching from the helical domain. In fact, the interaction of Lys545 with Asp421p85 compensates for the loss of the nSH2–helical interaction that the Glu545–Lys349p85a provides in the WT, and prevents nSH2 from detaching. Asp421p85a is located on the loop that links the nSH2 with the iSH2 domain. The interaction of Lys545 with Asp21p85 leads to the gradual detachment of the C-terminal region of the iSH2 and its i3x helix from the activation loop of the kinase domain that together keep it to the inactive conformation (Fig. S3A†). The third helix of the iSH2 domain (p85 residues 587–598), i3x, forms in the WT an interface with the activation loop that consists of hydrophobic interactions between Leu598p85a and Phe945p110a, and a hydrogen bond between Gln91p85a and Lys948p110a (Fig. S3B†). This interface is expected to stabilise the activation loop to the inactive conformation and deletions (Δ583–602), or truncations (p85–572STOP) of this section of the iSH2 domain are known to be oncogenic.16 In the mutant, the i3x helix moves further down the iSH2 domain retaining the hydrogen bond of Gln591p85a with Lys948p110a.

By extending our analysis of the unbiased MD simulations further away from the 545 position, a closer look on the salt-bridges formed between the charged residues on the surface of nSH2 and its neighbouring domains (Fig. S4†) shows that the introduction of Lys545 disrupts additionally the helical–nSH2 interaction Glu542p110a–Arg358p85a, as well as the nSH2–kinase interaction Glu342p85a–Lys948p110a. The latter, presumably, is one of the key interactions that keep the activation loop in the inactive conformation. The activation loop of the kinase domain is responsible for the recognition of PIP2 by PI3Kζ,17 and a catalytic scenario has been recently proposed, in which the 941KKKK944 polybasic stretch on the activation loop recognizes the PIP2 substrate, while His936p110a and His917p110a facilitate phosphorylation.14,25 Through analysis of the root mean square fluctuation (RMSF) of the activation loop over the course of our unbiased simulations, we see that the release of the nSH2–activation loop contacts, which we observe in two out of the three independent simulations of the E545K mutant, results in increased flexibility of the activation loop (Fig. S5†). We, thus, expect that this flexibility will allow the polybasic stretch to approach the γ-phosphate of ATP in the activated PI3Kζ.

It has been established that the effects of mutations can extend well beyond their specific points of localisation, resulting in long-range allosteric effects that overall translate into increased stabilisation of different states or of different regions assuming a functional role. Dynamical decomposition analysis has been used in the past to provide a simple yet valuable description of proteins dynamics, which can, in turn, determine the response to mutations.18,19 The results of the dynamical domain decomposition analysis of the WT PI3Kζ performed herein suggest that, at a subdomain level, the movement of the ABD domain is highly correlated with the C-terminal region of the iSH2 domain, while the N-lobe of the kinase domain is highly coupled with the C-terminal region of the helical domain (Fig. S6A and B†). In the case of the E545K PI3Kζ, the clustering of residues into eight subdomains suggests that the mutation increases the coupling of the motion of the C2 domain with that of the helical domain, and especially with the interfacial region between the nSH2 and helical domains (Fig. S6B†). Interestingly, when the residues are clustered into 11 subdomains, which is the number of domains with the second maximum quality score in the replicas where the mutation drives the release of the iSH2–activation loop regulatory contacts (rep0, rep2, Fig. S6A†), the residues of the Ser501p110a–Asp527p110a loop cluster together and independently of the helical domain (Fig. S6C†). This is in agreement with the observed sliding of the nSH2 that is seen in this set of simulations, which in turn increases the flexibility of the Ser501p110a–Asp527p110a loop. In the WT, decomposition into 11 domains leads to a clear division of the helical domain into two subdomains; one where the nSH2 domain binds to, and one where the N-lobe of the kinase domain is attached to (Fig. S6C†).

Multiple-walkers metadynamics simulations

The unbiased MD results suggest a mutant-driven activation, where the mutation leads to disruption of the iSH2 regulation of the conformational flexibility of the activation-loop of the kinase domain. Even at 1000 ns, however, MD simulations are too short to capture significant conformational changes. Since 1 μs of unbiased MD was insufficient to sample large conformational changes, we used multiple-walkers metadynamics
(metaD) simulations to enhance the sampling. The main free energy minima have been fully explored and a clear qualitative picture of the PI3Kα mechanism of E545K action can be drawn.

The free energy surface of the WT exhibits two minima that are equally populated within the error bar. Basin α1 (Fig. 2, Video S1†) corresponds to an ensemble of conformations that resembles the crystal structure. In this ensemble, the nSH2 is in contact with the helical domain even though the backbone hydrogen bond between Glu545 and Leu380p85α is not present in all conformations of the ensemble, and Glu545 interacts with Lys548p110α and Lys379p85α, and occasionally with Lys573p110α as well (Fig. S7A†). In this ensemble, the interaction of Lys419p85α with Asp549p110α of the loop that connects the nSH2 with the iSH2 domain, keeps the nSH2 bound to the helical domain even when the Glu545-Lys379p85α salt-bridge is broken. The activation loop is maintained in the inactive conformation due to salt-bridges between the side chains of Lys948p110α and Glu342p85α or Glu345p85α, and through a network of hydrogen bonds between Gln591p85α and Arg949p110α or Tyr947p110α (Fig. S7B†).

Surprisingly, the second basin in the free energy of the WT (basin α2, Fig. 2, Video S2†) corresponds to a previously unreported ensemble of conformations, where nSH2 has rotated around the helical domain (Fig. S7C†), as can be seen from the exposure of helix A (residues 339–347) to the solvent. This rotation makes biological sense as it may facilitate the binding of pY peptides or other tyrosine-phosphorylated signalling proteins since the cluster of residues (Arg340p85α, Arg358p85α, Ser361p85α, Thr369p85α) that stabilise the phosphotyrosine becomes solvent-exposed and therefore accessible for binding (Fig. S7C†). It should be noted that the rotation of the nSH2 domain does not affect the conformation of the activation loop in the kinase domain, which is held in the inactive conformation by the iSH2.

The main minimum on the free energy surface of the E545K mutant corresponds to a very broad energy basin in the defined contact map space (basin β1, Fig. 2, Video S3†). In this basin, the E545K PI3Kα is found in two main conformations. In the first conformation, the loop that connects helix B of nSH2 (residues 400–411p85α) with the iSH2 domain is flexible, Lys545 interacts mainly with Glu549p110α of the helical domain, and the salt bridge between Glu342p85α of the nSH2 domain and Lys948p110α of the activation loop is formed, keeping helix A in its original position. In the second conformation, Lys545 interacts with Asp421p85α while Lys419p85α points towards Glu347p110α similar to the conformation found in the unbiased MD. The interactions engaged by Lys545 may be the trigger of a conformational change in which the loop that connects helix B of nSH2 with the iSH2 domain pulls the iSH2 away from the activation loop. Specifically, the pulling effect bends the N-terminus.
terminal part of the iSH2, and the subsequent disruption of the inhibitory interactions between the iiz3 helix and the activation loop weakens the nSH2 domains-mediated regulation of the kinase activity. It should be noted that in the conformations of this basin, the backbone hydrogen bond between Lys545 and Leu380 is present, indicating that PI3Kz E545K can be activated without the nSH2 domain detaching from the helical domain.

The breakthrough discovery here is that nSH2 does not need to detach from the catalytic domain to release the nSH2 regulatory contacts with the activation loop upon the E545K mutation, and thus activate the kinase. The release of the iiz3–activation-loop contacts and the bending of the N-terminal part of the iSH2 along with a higher flexibility of the activation loop, expose helix A to the solvent (Fig. S8†), as also seen in the HDX-MS experiments. At the same time, the stronger nSH2–helical interaction in the predicted conformation (basin β1, Fig. 2) hinders pY binding, which could explain the reported insensitivity of E545K PI3Kz to pY activation.

The second minimum in the free energy surface of the E545K (basin β2, Fig. 2, Video S4†) corresponds to an ensemble of conformations, where the nSH2 domain has detached from the helical domain and moved away from the activation loop, in agreement with previous proposals. The nSH2 domain moves as a rigid body maintaining its overall folding and secondary structure elements and slides along the surface of the catalytic subunit rather than completely detaching from the protein surface into the solvent as was previously thought. It should be noted that in the absence of a pY peptide or a phosphorylated signalling protein in the simulated system, the nSH2 is stabilised only by non-specific interactions with the iSH2 domain, and therefore this basin is not highly populated.

Conclusions

Our simulations and free energy calculations show two different displacement paths resulting from the E545K mutation (Fig. 3).

One is the detachment of the nSH2 domain from the helical domain, and the other is the sliding along the helical domain. The first was also observed in our previous MD simulations and, as discussed above, might be the main event in the presence of the pY peptide that is expected to stabilise the detached nSH2 domain. When the nSH2 domain is detached from the helical domain, the activation-loop stays intact because its interactions with the isH2 domain are maintained, in agreement with HDX-MS experiments in the presence of pY, where E545K does not affect the catalytic subunit. The other effect of E545K is more surprising and directly affects the catalytic subunit. The newly formed contacts of the Lys545 with residues of the linker that connects the nSH2 with the iSH2 domain trigger a conformational change in the iSH2 domain that results in the release of the iSH2–activation loop regulatory contacts and affect the activity allosterically. The perturbation around E545K is propagated to the isH2 domain through the loop that connects the two domains, and the breaking of the inhibitory contacts between iSH2 and the activation-loop “unlocks” the activation-loop, which is expected to be free to adopt active-like conformations, especially in the presence of PIP2. Such a mode of action is in line with recent observations in several multi-domain proteins, showing that linkers can propagate mechanical signals allosterically between domains and facilitate correlated movement.

Both events, that is the sliding of nSH2 around the helical domain and its detachment from the helical domain result from the E545K mutation (Fig. 3). However, the first effect is probably the most relevant in terms of the deregulation of the catalytic activity as it exerts an allosteric effect on the catalytic subunit triggering the release of the nSH2 regulatory contacts. This does not exclude the possibility of both events happening sequentially; first, the release of the iSH2–activation loop regulatory contacts and then the detachment of nSH2, although more experimental data is needed to validate this hypothesis. The proposed mechanism and the new conformation reported here provide also a different and more complete interpretation of existing experimental data.

Methods

The structure of the WT PI3Kz heterodimer was retrieved from the Protein Data Bank (PDB ID: 4OVU†). Missing residues and the E545K mutation were modelled using Prime (Schrödinger suite). Following system setup, the WT and mutant proteins were solvated and equilibrated as described in the ESI. The CHARMM36m force field was used for the protein and the CHARMM TIP3P model for the water molecules. A total of 2 μs of unbiased MD simulations were performed for each system with the GROMACS 2016.4 MD engine to characterise the local dynamics.

Subsequently, we used multiple walkers metadynamics simulations to characterise the effect of the E545K mutation to the nSH2-mediated autoinhibition and calculate the corresponding free energy surfaces (FESs). The FESs of the WT and mutant PI3Kz were reconstructed as a function of two collective variables (CVs) after 400 ns of simulation time per system. CV1

Fig. 3 Observed displacement paths upon the E545K mutation.
is the distance between the centres of mass of the nSH2 and helical domains, and CV2 is the distance in terms of contact maps from a reference state in which the nSH2 domain has detached from the helical domain (see ESIF for more details on the setup). A funnel-shaped potential around the nSH2 domain was used to aid the convergence of the free energy (Fig. S9f).

The quasi-rigid dynamic domain decomposition was performed using the SPECTRUS method, a dimensionality reduction approach (spectral clustering) that allows the identification of dynamical domains in protein complexes.\(^9,24\) The clustering algorithm computes the inter-residue distance fluctuation matrix on frames extracted from an MD simulate, which is used to define the subdivision into domains. Then, a quality score is used to measure the robustness of the domain separation, \(i.e.,\) how much the calculated clusters are overlapping and distinct from each other. This score results from computing the ratio between the distance of each residue to its own cluster centre and the distance to the second closest cluster centre. The local maxima of the reciprocal of this quantity, averaged over all residues, mark the decomposition into well-defined clusters and allow the \textit{a posteriori} identification of the optimal number of clusters (domains) to be considered. Here, we used the default setup of the SPECTRUS method with 1000 iterations of the k-medoids algorithm and 10 Å for nearest neighbours cutoff.

**Conflicts of interest**

There are no conflicts to declare.

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**Notes and references**