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The phosphorylation specificity of B-RAF^{WT}, B-RAF^{D594V}, B-RAF^{V600E} and B-RAF^{K601E} kinases: An *in silico* study

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ABSTRACT

Phosphorylation of the B-RAF kinase is an essential process in tumour induction and maintenance in several cancers. Herein the phosphorylation specificity of the activation segment of the wild type B-RAF kinase and the B-RAF^{D594V}, B-RAF^{V600E} and B-RAF^{K601E} mutants was examined by molecular dynamics (MD) simulations and GRID molecular interaction field analysis. According to our analysis, Thr599 and Ser602 were the only residues in the activation segment in B-RAF^{WT} that were well exposed to ATP binding, which is in agreement with the experimental results, and provide a molecular basis of the observed phosphorylation. The phosphorylation specificity was altered significantly for each of the three different mutants studied due to the large conformational changes and subsequent alterations in the electrostatic forces between several residues for each of these mutants. Thus the analysis revealed limited phosphorylation potential of the non-active B-RAF^{D594V} mutant and several potential ATP binding sites were identified for the highly active B-RAF^{V600E} mutant.

The Lys601 residue, which is specific to RAF and not present in the activation segment of other similar kinases, was identified to potentially be of major importance to the observed differences in the phosphorylation specificity of the mutants. Our results indicate that Lys601 might be a specific ATP coordinating residue, contributing to the B-RAF phosphorylation specificity. The overall results can be helpful for the understanding of the B-RAF phosphorylation processes on a molecular level.

1. Introduction

The RAF–MEK–ERK signal transduction cascade is a conserved protein pathway that regulates cell growth, differentiation, and proliferation in response to external stimuli (growth factors, cytokines, or hormones) [1]. When B-RAF is activated it activates the protein kinase MEK, which in turn activates a third protein kinase called ERK. As phosphorylation of the B-RAF kinase is a vital process to mediate it's activation, better understanding of ATP binding in the wild type B-RAF and key mutations is essential. Clinically, it has been determined that ~66% of melanomas, ~36% of thyroid tumours, and ~10% of colon cancers in humans can be correlated with mutations that have occurred in the B-RAF kinase domain [2]. Thus, B-RAF has been considered as an attractive new drug target.

It has been experimentally established that the activation of wild type B-RAF (B-RAF^{WT}) requires phosphorylation of the activation segment (named A-loop here) at the Thr599 and/or Ser602 residues [3]. It has been suggested that these two residues are those most exposed to ATP molecules [4], but there is still neither available experimental nor theoretical arguments that support this hypothesis. It was proposed that the V600E mutation mimics the phosphorylation step and can activate MEK directly and subsequently stimulate ERK without phosphorylation taking place [2,5]. GLU600 is considered to acts as a phosphomimetic between the Thr599 and Ser602 phosphorylation sites, inducing conformational change in the protein structure [6]. It was speculated that most of the other activating B-RAF mutants mimic the phosphorylation step as well [5,6]. However, experimental data for the phosphorylation specificity of the activation segment is only available for the wild type B-RAF, and thus sufficient data to support this hypothesis is not yet available. Moreover, it has been shown that the activated B-RAF^{V600E} binds to an Hsp90–cdc37 protein complex. It is reported that this process is required for the B-RAF^{V600E} stability, function and the evolution of the melanomas and the other tumours that depend on this mutation [7].

A significant number of mutations of B-RAF are reported; most of them with elevated activity compared to the basal one, but four mutants have impaired kinase activity [5]. Three of the impaired activity mutants are only capable of inducing ERK phosphorylation through heterodimerization with C-RAF. The fourth mutant, B-RAF^{D594V}, acts like a kinase-dead mutant and cannot bind to C-RAF. Its role in tumorigenesis remains to be elucidated [8].

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Asp594 is a key catalytic residue for many kinases, and thus it is not surprising that B-RAF^{D594V} is a loss-of-function mutant [9,10]. However, the impact of the D594V and several other mutations on the conformation of residues that can potentially interact with ATP, such as Lys483, Glu501 and Asp594, and how the different mutations influence the phosphorylation specificity and the activation of the different mutants, is yet unclear. Also, the role of phosphorylation in the activation of strongly activating B-RAF mutants, such as V600E and K601E, is not well understood [5,11].

The crystal structures of the inactive B-RAF^{WT} and B-RAF^{V600E} kinase binding domains in a complex with the inhibitor Sorafenib (BAY-439006) have been published [5] and recently the structures of the active B-RAF form were solved as well [12–14], adding to the understanding of these type of kinases. However, a significant portion of the activation segment, which plays an essential role in phosphorylation and kinase activation, was not solved in any of the available structures.

The purpose of this study is to analyse the phosphorylation specificity of B-RAF^{WT} and three key mutants (B-RAF^{V600E}, B-RAF^{D594V} and B-RAF^{K601E}) and to gain improved understanding of the underlying molecular basis for experimentally measured activity deviations. The full structural models of these four B-RAF forms were based on the available crystal structures, supplemented by models for the missing A-loop portions [15,16]. The structures were refined by 15 ns molecular dynamics simulations, and the phosphorylation specificity of each kinase was analysed with the GRID method. Previous studies of the effect of the different mutations on especially the electrostatic interactions and on the formation of networks of hydrogen bonds within the binding cavity were used to provide additional support to our results [15,16].

2. Methods

2.1. Preparation of protein structures

Before carrying out the MD simulations and the GRID analysis, the structures of B-RAF^{WT}, B-RAF^{V600E}, B-RAF^{K601E} and B-RAF^{D594V} were prepared as previously described [15]. For the two B-RAF forms, B-RAF^{WT} and B-RAF^{V600E}, for which crystal structures were available in the protein data bank [17] (pdb codes: 1uwh and 1uwj), the structures were prepared by removing the inhibitor Sorafenib and keeping the crystal waters. Those A-loop residues not solved in the crystal structures, residues 601–612 and 603–614 in B-RAF^{WT} and B-RAF^{V600E}, respectively, were modelled with the Modeller software [18]. The mutants B-RAF^{K601E} and B-RAF^{D594V} were constructed by substituting the original residues of the crystal structure of B-RAF^{WT} with the appropriate amino acids, respectively. The A-loop region was modelled and refined in the same way as for the two other B-RAF forms.

2.2. Molecular dynamics

To obtain sufficiently accurate structures for the GRID analyses, we used structures obtained from a series of 15 ns MD simulations of the wild type kinase and the studied B-RAF mutants [16]. Before initiating the MD simulation, the complexes were prepared with the Amber 9 software [19] and subsequently the MD simulations were carried out with NAMD v.2.6 [20]. The simulated systems were placed in the centre of a truncated octahedron simulation box filled with water molecules, which were represented with the SPC/E water model [21]. The buffering distance was set to 10 Å. Counter ions were added to maintain the electro neutrality of the complexes. The Amber 2003 force field was employed for the simulations [19]. All systems were energy minimized in two steps. First only the waters and ions were minimized for 2000 steps keeping the protein fixed. A second minimization was then performed on the whole system for 8000 steps with the conjugate gradient method to convergence criterion of 0.5 kcal mol^{-1} Å^{-1}. Initially, the simulation complexes were gradually heated from 0 to 300 K for 30 ps (NTV), further equilibrations for a 200 ps were performed, followed by production runs of 15 ns (NTP). Langevin dynamics was utilized to keep a constant temperature. Constant pressure of 1 atm was imposed using the hybrid Nosé–Hoover Langevin piston method with a decay period of 200 fs and a damping time-scale of 50 fs. The non-bonded cut-off was set to 12.0 Å. The SHAKE algorithm was applied for all bonds involving hydrogen atoms [22]. A 2 fs integration time step was used, and long-range electrostatic interactions were treated with the particle-mesh Ewald (PME) method [23]. Data were collected every 500 steps, i.e., every 1 ps. The calculation of the root-mean-square deviations (RMSD) and the corresponding standard deviations (SDs) were carried out with the VMD package and its Tcl routine [24].

2.3. GRID calculations

In order to indentify the possible ATP binding sites and to obtain detailed understanding of the forces contributing to the phosphorylation specificity of the studied B-RAF forms, a series of calculations were performed with the GRID program, version 22 [25,26]. The three-dimensional structures for each of the B-RAF forms obtained from the 15 ns MD simulations [16] were imported into the GRID program, and the non-covalent interactions between the proteins residues and small chemical groups, so-called probes, were calculated. By generating a grid net in the proteins binding pocket and placing a probe at each grid point, a set of interaction energies between the probe and the surrounding protein residues are calculated.

Thus, in order to indentify those residues that might react with a phosphate group provided by ATP, i.e. residues that can potentially undergo phosphorylation, a PO_4H group was used as probe. To obtain greater understanding on how the electrostatic forces induced by the ATP molecule affect ATP transport and binding, a negative charge of −3 was used as probes as well. These two probes where thus used to generate a virtual reaction between ATP and the protein residues in order to mimic the phosphorylation process within the B-RAF binding site.

GRID maps were used to identify and graphically illustrate the favoured ATP binding sites of the kinases. A cut-off value was introduced, and regions with protein–probe interactions energies that are more negative or equal to −15.0 kcal/mol are shown. The graphs were then used to identify residues that exhibit strong protein–probe interactions.

For each the B-RAF forms, the GRID calculation was done by centring the box on the proteins centre with sufficient dimensions to accommodate the proteins. A grid box of density one point per 1 Å was used, and the interactions between the protein and the two selected probes were evaluated by placing the respective probes at each grid point.

3. Results and discussion

3.1. Molecular dynamics

Detailed analysis of the MD simulations that form basis for the GRID calculations is presented in a separate publication [16]. The mean RMSD of the backbone proteins atoms during the simulation period for B-RAF^{WT}, B-RAF^{V600E}, B-RAF^{K601E} and B-RAFD^{D594V} atoms were 1.70 Å (SD = 0.13), 2.57 Å (SD = 0.27), 2.09 Å (SD = 0.26) and 1.82 Å (SD = 0.26), respectively (see Fig. 1A in Ref. [12] for details).