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Article

Analyses of a Select Set of BRAF Mutants and Implications for Their Mechanistic Action as Drivers of Carcinogenesis

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Abstract: Oncogenic BRAF mutations represent a variety of amino acid changes at multiple locations within the BRAF kinase domain. Their classification, as class I, II or III, depends on their RAS dependency, their dimerization status in their signalling state and kinase activity. However, many of these mutants have not been fully characterised for their *in vitro* oligomeric state and kinase activity. Additionally, their interaction with the Hsp90-CDC37 complex, an important component in their stability and activation, is poorly defined. As these properties likely affect the exact mechanism by which these mutants drive carcinogenesis it is of clinical importance that such a characterization is undertaken. Here we report the purification and characterization of a select number of BRAF-mutant proteins so that we can better understand their mode of action. We find that the purified class-II E586K-mutant kinase domain of BRAF is active in its monomeric state, whereas the class-II mutant L597V is, in contrast, an inactive monomer. We also find that purified T599W, where the tryptophan substitution eliminates an activating-phosphorylation site, was active for kinase activity. Consistent with the monomeric state of these mutant BRAF proteins, they interacted with CDC37, although T599W did show somewhat reduced affinity. Our findings, question the strict classification of specific BRAF mutants and suggest further investigations are required to fully understand their carcinogenic mechanism.

Keywords: Hsp90; CDC37; BRAF; mechanism; chaperone; cochaperone

1. Introduction

Protein kinases regulate key eukaryotic signalling pathways involved in processes such as cell survival, metabolism, proliferation, cell migration, differentiation and the cell cycle [1]. Consequently, the regulation of protein kinases is of paramount importance as dysregulated activity often leads to cell transformation and cancer [2], and this is particularly relevant for the BRAF V600E-mutant (B-Rapidly Accelerated Fibrosarcoma, V600E mutant), which is considered the most highly mutated protein that drives carcinogenesis [3].

BRAF is known to exist as an ensemble of conformations due to its metastable state [4,5], which in turn defines its dependency on the Hsp90-CDC37 (heat shock protein 90–cell division cycle 37) complex. CDC37 is a cochaperone of Hsp90 and acts as a kinase specific adaptor that delivers BRAF and other protein kinases to the Hsp90 complex [6]. The classical mechanism for BRAF activation occurs by translocation of the cytoplasmic Hsp90-CDC37-BRAF complex to the cell membrane [7], where BRAF is then activated by a RAS-dependent mechanism that also involves 14-3-3 [8]. Inhibition of Hsp90 by geldanamycin leads to a rapid dissociation of both the Hsp90-BRAF and RAS-BRAF multimolecular complexes, increased proteasomal degradation of BRAF and to a decrease in translocation of BRAF to the plasma membrane [9–12]. In the absence of RAS the most

populated state of BRAF appears to be the autoinhibited form that is further stabilised by an interaction with 14-3-3, which binds directly to the phosphorylated amino acid residues Ser 365 and 729 (pSer 365 and 729) of BRAF [8]. In the presence of activated RAS, however, there is a shift from the inactive state to an active BRAF signalling complex [13]. The general consensus is that the interaction of RAS with the inactive BRAF-14-3-3 complex displaces 14-3-3 from pSer 365 allowing either homodimerization of BRAF or heterodimerization with other RAF molecules [14], The dimer form of BRAF then remains tethered through the pair of pSer 729 residues each bound to one molecule of the dimeric 14-3-3. Finally, the dimerization of BRAF by RAS allows the *cis*-autophosphorylation of Thr 599 and Ser 602 of the BRAF activation loop (the $D^{594}FG$ motif to Gly 615) [14,15]. Such phosphorylation is critical for inducing and stabilising a conformational change that leads to alignment of the C- (catalytic) and R- (regulatory) hydrophobic spines of the kinase domain [16–18], thus promoting ATP uptake, full catalytic kinase activity and consequently MEK-1 phosphorylation [16,19]. In contrast to wild type BRAF, the Hsp90-CDC37-BRAF V600E complex appears to be not only more abundant than the 14-3-3 complex, but also more active [20]. Consequently, the partitioning of BRAF between the 14-3-3-RAS and the Hsp90-CDC37 complex is altered due to the V600E mutation. Understanding, how each BRAF mutation alters its mechanism of activation and stability is a crucial point to understand for patient treatment.

BRAF mutants that drive oncogenesis consist of three classes [21–24]. The class I Valine 600 mutations of BRAF are defined as RAS-independent active monomers. Consequently, they are insensitive to ERK1/2 and SOS feedback inhibition. In contrast, Class II mutants function as active or partially (or intermediate) active dimers, that are consequently RAS-independent. Class II mutants therefore escape feedback inhibition through the ERK and phosphorylation of SOS, a modification that downregulates BRAF activity. In contrast, the RAS dependent BRAF class III mutants are kinase dead, but increase signalling through the MAPK pathway due to enhanced RAS binding and subsequent CRAF activation. While this classification aids our understanding of the mechanistic action of BRAF mutants, there are cases where a clear and detailed characterization that supports their proposed mechanism is lacking. For example, some reports suggest that partially or intermediate active BRAF mutants also associate with RAS [25].

Recently, we purified the class II L597R-mutant kinase domain of BRAF and found that although it appeared to be dimeric *in vitro*, it lacked kinase activity and an ability to interact with CDC37 [12,20,26,27]. Furthermore, it was recently shown that other non-V600E mutations have reduced ability to associate with the Hsp90-CDC37 complex, which supports our findings that dimerization limits interaction with the Hsp90-CDC37 complex [28,29].

Interestingly, the dimeric state of L597R could transition back to a monomeric form by introducing the V600E mutation, but the double mutant lacked kinase activity. This suggests that L597R might not be activatable. As an inactive homodimer, the mechanism by which L597R drives carcinogenesis needs to be re-evaluated. Mechanisms that might allow L597R to drive carcinogenesis could include a RAS dependent or independent process (utilizing 14-3-3 alone). Thus, L597R might form a heterodimer with ARAF or CRAF, where the non-BRAF partner protein becomes the signalling component of the heterodimer. In support of this idea, some class II BRAF-mutants show elevated levels of coincidence with RAS mutations [30]. Additionally, for some BRAF mutations coincidence with NF1 alterations has been reported [31]. However, an alternative mechanism for BRAF activation may occur via 14-3-3-mediated homodimerization, but this would normally operate if the BRAF mutant could achieve activation, which appears not to be the case for L597R, since it lacked activity when combined with the constitutively activating V600E mutation. In such a case, it would appear to then be dependent on heterodimerization with CRAF or ARAF. Recent structural and biochemical work has shown that binding of the 14-3-3 scaffold protein to phosphorylated C-terminal motifs on BRAF induces it to dimerize, which is sufficient for BRAF activation even when RAS is not involved [32]. The key requirement here for activation is 14-3-3–mediated dimerization rather than a dependency on the RAS-binding N-terminus or regulatory DTS region (distal tail segment, residues 742–748). Normally the DTS region is required to complete the C-spine of the receiver kinase and therefore promote catalysis in the dimer partner BRAF molecule. A recent cryo-EM structure of the BRAF-14-3-3 tetrameric complex shows that the “distal tail segment” (DTS, residues 742–748) of one BRAF molecule is bound to the BRAF ATP-binding site of its dimer partner. However, in the tetrameric BRAF-14-3-3 complex, BRAF adopts a catalytically active form comparable in activity to the constitutively active V600E mutant. In this scenario the pS365 site is dephosphorylated and an active dimer of BRAF is stabilized by the binding of two pS729 sites, one form each BRAF monomer bound to a one molecule of a 14-3-3 dimer [33,34]. The tetrameric state of this complex counteracts the effect of endogenous cellular ATP in breaking RAF dimers, and increases catalytic activity for the phosphorylation of MEK [33]. This is similar to “paradoxical activation”, a transactivation mechanism of one BRAF molecule by the binding of a small-molecule inhibitor to its partner molecule [35–37]. Furthermore, in this scenario, BRAF may also activate CRAF through a mechanism involving 14-3-3-mediated heterodimerization and CRAF transphosphorylation, which leads to activation of the BRAF–

CRAF–MEK–ERK cascade [38]. A clear understanding of the precise mechanisms underlying RAS-independent activation of BRAF mutants is of course clinically important. Therapeutic strategies targeting BRAF driven carcinogenesis must account for 14-3-3–mediated activation and dimerization pathways, regardless of RAS status, as these mechanisms contribute to the paradoxical activation of BRAF.

In this study, we investigated the dimeric state of a select number of BRAF mutants. We characterise the class II mutant L597V, and ask if it shares similar characteristics to our previously characterised L597R mutant, which presented, *in vitro*, as an inactive kinase dimer. The L597 mutations are found in a variety of cancers that include melanoma, lung adenocarcinoma, colorectal cancer, ovarian cancer, thyroid cancer to name but a few [39–42]. We also characterised E586K, another class II BRAF-mutant [43]. Examples of E586K driven carcinoma have been reported in colorectal adenocarcinoma, cutaneous melanoma, and non–small cell lung cancer [44–47]. To compare, we also characterised D594V, a known class III BRAF mutant [23]. D594V has been reported in non-small cell lung cancer and colorectal cancer cancers including metastatic cases [48–50]. Finally, we investigate the effect of a tryptophan mutation at T599, a known phosphorylation site for the full activation of BRAF. Although T599W is not a known to be a driver of carcinogenesis, the T599I mutation which possesses partial kinase activity, is a known driver for cancer [43,51]. However, we wondered whether a bulky residue such as tryptophan would also be catalytically active for kinase activity.

2. Materials and Methods

2.1. Protein Expression and Purification

Human C-terminally His-tagged CDC37 and N-terminally GST-tagged sBRAF kinase domain (a soluble version of BRAF, residues 423–723 and referred to as BRAF throughout the manuscript) [52], including mutant forms, were obtained from Genscript. Proteins were expressed and purified as previously described [26] and dialysed against 20 mM Tris/HCl (MERCK, Calbiochem, catalogue No. 648317, Darmstadt, Germany), pH 7.5, containing 1 mM EDTA (MERCK, Sigma Aldrich, catalogue No. E5134-1KG, Darmstadt, Germany), and 200 mM NaCl (MERCK, Sigma Aldrich, catalogue No. S9888-5KG, Darmstadt, Germany) and stored frozen at -20 °C**.** MEK-1 was purified as previously described [12] and represented a C-terminally His-tagged fragment of approximately 35.6 kD.

2.2. Isothermal Titration Calorimetry and Kd Determinations

Heat of interaction was measured on an ITC200 microcalorimeter (Malvern) under the same buffer conditions (20 mM Tris, pH 7.5, containing 1 mM EDTA and 200 mM NaCl). Aliquots of CDC37 construct at 120 μM were injected into 10 μM of BRAF mutant at 20 °C, all except for the T599W and CDC37 interaction, where we used 100 μM of CDC37 and 5 μM T599W mutant protein. In general, we found T599W less soluble and hence we limited the final concentration of the protein. Heats of dilution were determined by diluting injectant into buffer. Data were fitted using a curve-fitting algorithm (OriginLab Cooperation, Microcal Origin, version 7.0, Northhampton, MA, USA).

2.3. BRAF Kinase Assays

The kinase activity of BRAF and its mutants were determined by using a MEK-1 phosphorylation assay consisting of either 9 or 35 μM BRAF, 6 mM $MgCl_2$ (MERCK, Calbiochem, catalogue No. 442611-M, Darmstadt, Germany), 5 mM ATP (MERCK, Sigma Aldrich, catalogue No. A7699-5G, Darmstadt, Germany), 1 μg inactive MEK-1 (C- terminally His-tagged) in a total volume of 40 μL buffer consisting of 20 mM Hepes pH 8 (MERCK, Sigma Aldrich, catalogue No. 54457-250G-F, Darmstadt, Germany), 1 mM DTT (MERCK, Sigma Aldrich, catalogue No. D9779-25G, Darmstadt, Germany) and 100 mM NaCl. Reactions were incubated at 30 °C for 60–180 min for 35 μM BRAF and 240 to 480 min for 9 μM BRAF reactions. 10 μL samples were taken for western blot analysis using anti-phospho MEK-1/2 (residues 218/222 and 222/226, MERCK, catalogue No. 05-747, Darmstadt, Germany) as the primary antibody and rabbit secondary HRP (Cyvita, catalogue No. NA934-1ML, Marborough, MA, USA) both at a 1/5000 dilution. Detection was carried out using a Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, catalogue No. 32106, Waltham, MA USA).

2.4. Molecular Mass Determination

A Superdex 200 Increase 10/300 GL gel-filtration column equilibrated in 50 mM HEPES pH 7.5, 300 mM NaCl, 0.5 mM TCEP (MERCK, catalogue No. C4706, Darmstadt, Germany) and 1 mM EDTA was used to

determine the relative molecular mass of BRAF proteins. The gel-filtration standards used to calibrate the column included β-amylase (200 kD), conalbumin (75 kD), ovalbumin (44 kD) and carbonic anhydrase (29 kD) selected from two kits (GE Healthcare, catalogue No. 28-4038-41, Chicago, Illinois, IL, USA and MERCK, Sigma Aldrich, catalogue No. MWGF1000, Darmstadt, Germany). BRAF mutants are considered monomeric if they migrate close to their calculated monomeric size of approximately 34 kD, while L597R migrated with a relative molecular mass of 57 kD, was considered dimeric.

3. Results

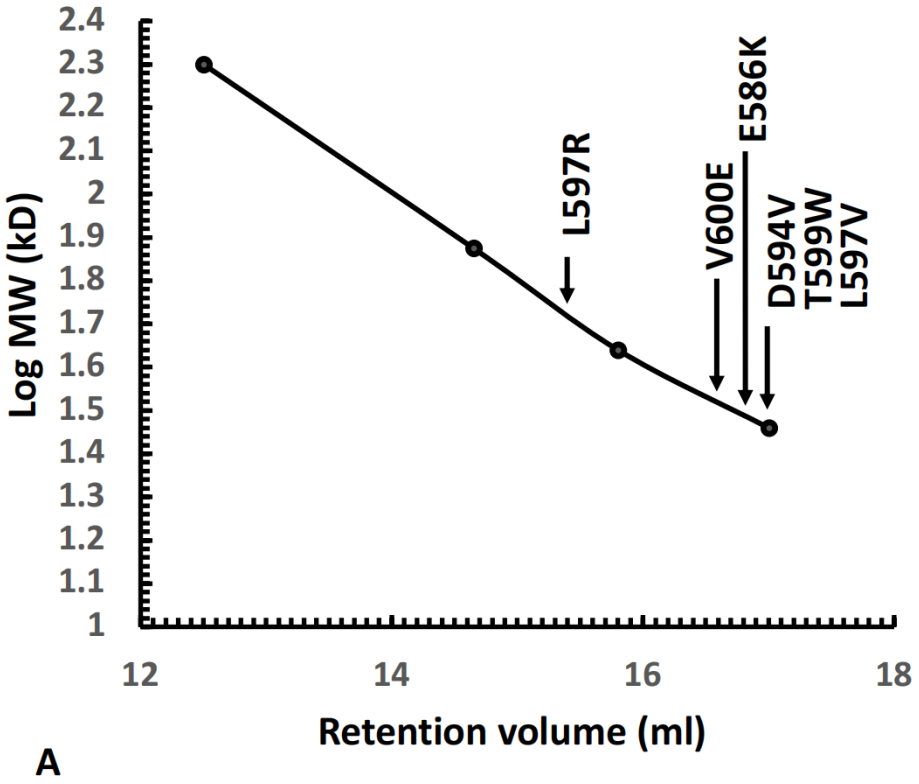
3.1. The Oligomeric State of the E586K, D594V, L597V and T599W Mutants of BRAF

Class-II BRAF mutants act, *in vivo,* as kinase-active dimers with at least partial kinase activity [21]. Recently, we showed that the purified L597R class-II mutant of BRAF was dimeric, but surprisingly lacked kinase activity. Furthermore, it appears that the dimeric structure of L597R prevents its interaction with CDC37. In support of this, by introducing the V600E mutation into the L597R mutant we were able to convert the mutant protein into its monomeric state that was then able to bind CDC37. However, this double mutant remained inactive for kinase activity even though it possessed the constitutively activating V600E mutation [26]. We therefore wondered whether the L597R mutation was characteristically different to other mutations at the L597 position. Consequently, we purified and characterised L597V to determine its oligomeric state, its ability to bind CDC37 and whether it possessed any kinase activity. Furthermore, we purified and expressed additional BRAF mutants to evaluate their characteristics. These included two activation segment mutants, namely D594V (a class III BRAF-mutant), and T599W, which eliminates the phosphorylation site within BRAF required for its full activation. Lastly, we purified and characterised the class II mutant E586K, which occurs in the connecting region between the catalytic loop (represented by the HXD motif; H^{574}RD in BRAF) and the activation loop (the D^{594}FG motif to Gly 615) of BRAF.

Using gel-filtration chromatography, we found that D594V, E586K, L597V and T599W all appeared to be monomeric relative to our V600E monomeric (relative molecular mass of approximately 34 kD) and L597R dimer control proteins, the later of which displayed a relative molecular mass of 57 kD, suggesting dimerization (Figure 1) [26]. We were, however, surprised to find that L597V behaved as a monomer on gel-filtration, unlike the related L597R mutation (Figure 1). Since L597V has been shown to be sensitive to the BRAF R509H mutation, this suggests that *in vivo,* dimerization of L597V remains a mechanism through which L597V drives carcinogenesis [22,36]. Consequently, our results suggest that mutations at the L597 amino acid position of BRAF, can have different effects on the *in vitro* and *in vivo* oligomeric state of BRAF. In other words, unlike L597R, other class-II mutants, such as E586K and L597V, may not display a dimeric state as purified proteins, but nonetheless may achieve this in a cellular context.

3.2. The Kinase Activity of the E586K, D594V, L597V and T599W Mutants of BRAF

We next decided to test the kinase activity of our BRAF mutants. To detect BRAF activity, we used the BRAF phosphorylation of MEK-1 as previously described [26]. Our V600E control was active and L597R was inactive as previously reported (Figure 2). Similarly, to L597R, L597V was also inactive and remained so as the L597V-V600E double mutant, even though it possesses the constitutively activating V600E mutation (Figure 2B) [26]. In contrast, to our findings, L597 mutants such as L597V have been reported to show partial or intermediate kinase activity [53–55]. Furthermore, the lack of L597V-V600E kinase activity and its monomeric state *in vitro* suggests the *in vivo* dimerization of L597V with other RAF homologues *in vivo*. However, as expected, the class-III D594V mutant was inactive (Figure 2B), which was consistent with its class classification. In contrast, the class-II mutant, E586K, showed significant kinase activity, even though it appeared to be monomeric as a purified protein (Figures 1B and 2C). The E586K-V600E mutant was also shown to be active (Figure 2C). While E586K may signal as a dimer in cells, its intrinsic kinase activity—even in the absence of cellular dimerization mechanisms—may also have clinical relevance. Clearly, more research needs to be conducted to fully understand the situation in cells for E586K driven carcinogenesis.



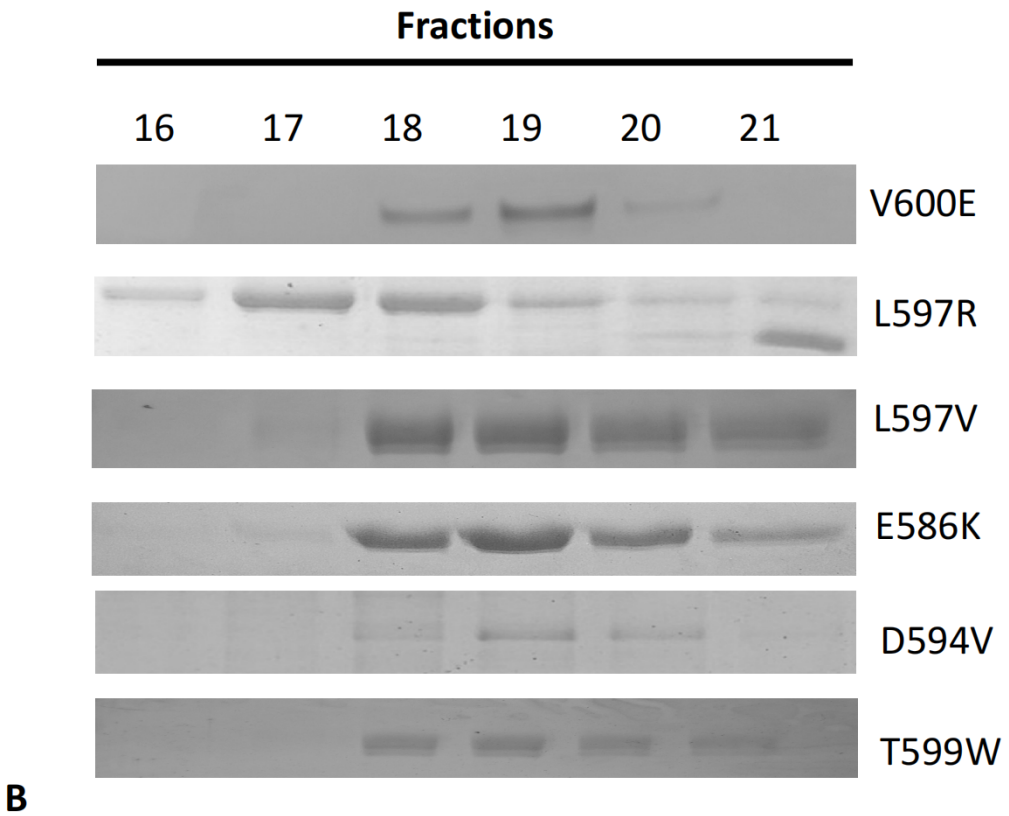


Figure 1. Estimation of the relative molecular mass of BRAF mutants. (**A**), Estimation of the relative molecular mass of BRAF mutants by gel-filtration chromatography. (**B**), SDS-PAGE gels showing the elution fraction from gel-filtration of the BRAF mutants. Proteins eluting with a relative molecular mass of approximately 34 kD are considered as monomer, whereas L597R eluted with a relative molecular mass of 57 kD, was considered dimeric.

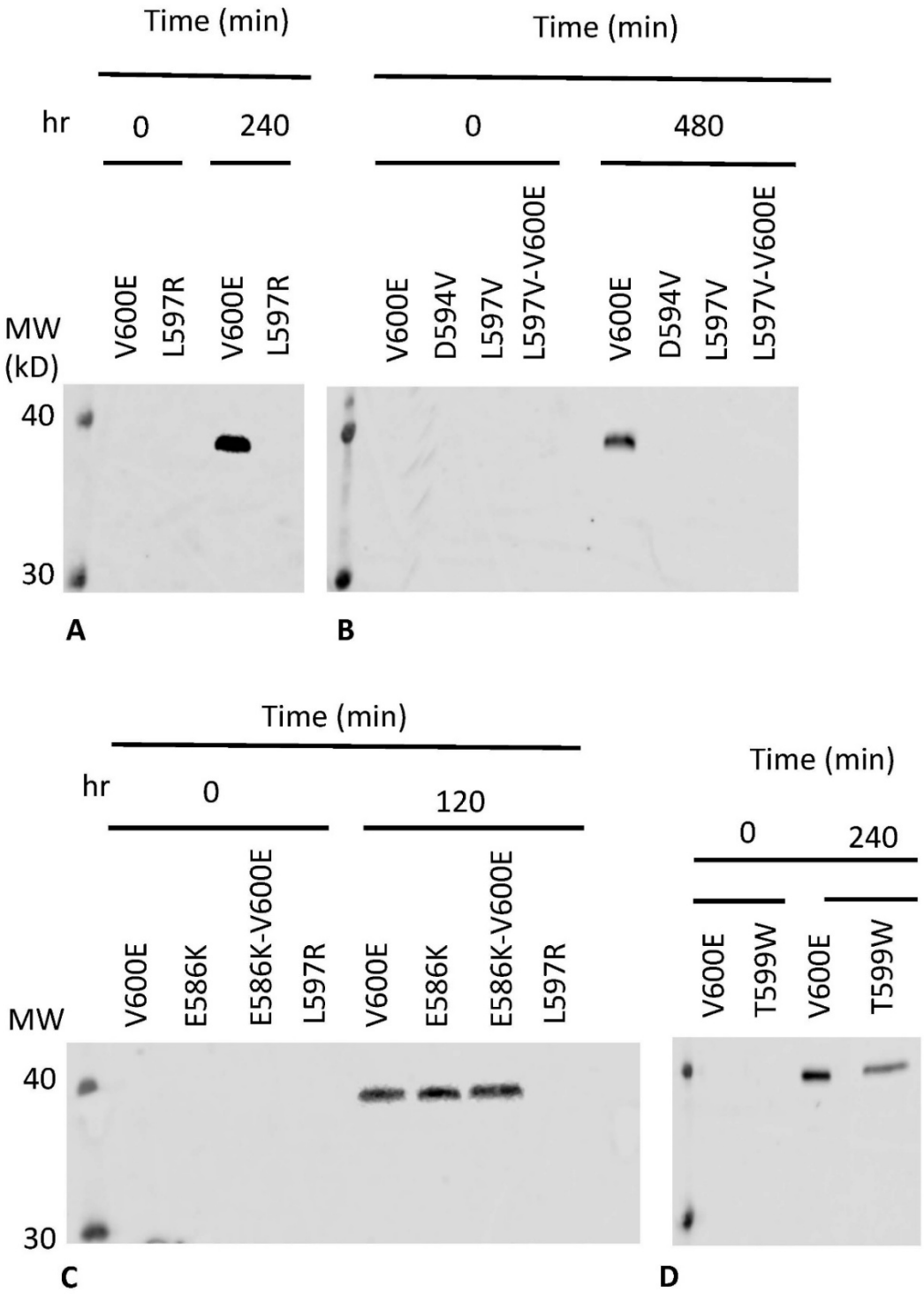


Figure 2. MEK-1 phosphorylation assays using mutant kinase domains of BRAF. (**A**) The kinase activity of V600E and L597R control proteins, showing active V600E and inactive L597R. (**B**) Kinase assays showing that D594V, L597V and L597V-V600E are inactive for kinase activity. (**C**) Kinase assays showing that E586K and E586K-V600E are active for kinase activity. (**D**) Kinase assays showing T599W is active for kinase activity. MW, molecular weight markers (kD).

3.3. The Activation Loop T599W BRAF-Mutant Is an Active Kinase

Although the T599W mutation has not been previously seen as a driver of carcinogenesis, to the best of our knowledge, it does however eliminate a critical regulatory site that is phosphorylated for the full activation of BRAF activity [15], and is a known site of mutation. For example, T599I has been seen to drive carcinogenesis and is regarded as a class-II mutant, which possess partial kinase activity [50]. Here, we find that T599W displayed kinase activity as a monomeric protein since it was able to phosphorylate MEK-1 (Figure 2D). The results for

T599W suggest that T599I might also be active as a monomer, and like E586K the role that T599I plays as an active-monomeric-kinase domain in driving cancer needs further investigation.

3.4. Monomeric BRAF-Mutants Interact with CDC37

Previously we had shown that a variety of activation loop mutants did not disrupt interaction with CDC37 [26]. These included BRAF L597A, F595A, F595V, D594V and the double mutants L597R-V600E and T599W-V600E, as well as a triple mutant T599E-V600E-S602D. To date, the only BRAF mutation shown to disrupt interaction with CDC37 is L597R, which appears to be due to its stable dimeric state [26]. To build on this, we decided to test the binding to CDC37 of the BRAF mutants T599W, L597V and the double mutant, L597V-V600E. We found that all these mutants interacted with CDC37 (Figure 3), which was consistent with their monomeric state (Figure 1). However, the interaction between T599W and CDC37 was compromised to some degree, with a much-reduced *K*d of 2.4 μM, compared to BRAF V600E, which had a *K*d of 0.63 μM. It is noteworthy, however, that the double mutant T599W-V600E, had a *K*d (0.23 μM) similar to that of the V600E control (0.63 μM) [26]. The reason behind the reduced affinity between T599W and CDC37 remains unresolved, and requires further investigation, but ultimately represents a conformation that stabilises BRAF against CDC37 interaction.

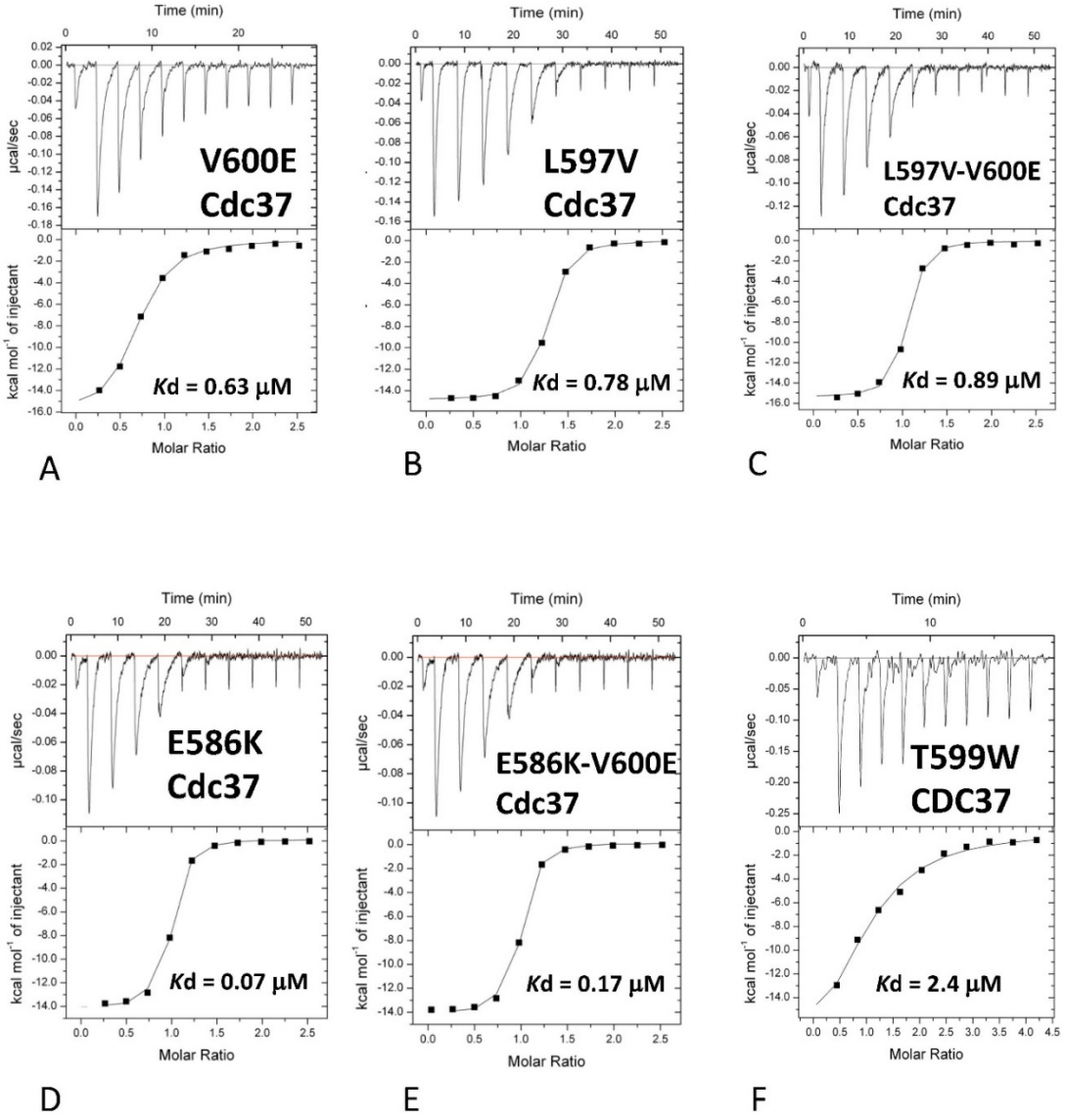


Figure 3. ITC of BRAF mutant with CDC37. Aliquots of CDC37 construct at 120 μM were injected into 10 μM of BRAF mutant at 20 °C, all except for the T599W and CDC37 interaction, where we used 100 μM of CDC37 and 5 μM T599W mutant protein. The protein pairs used and the *K*d for the interaction are shown in each panel. (**A**) The CDC37 interaction with BRAF-V600E; (**B**) The CDC37 interaction with L597V; (**C**) The CDC37 interaction with L597V-V600E; (**D**) The CDC37 interaction with E586K; (**E**), The CDC37 interaction with E586K-V600E and (**F**) The CDC37 interaction with T599W.

5. Discussion

While *in vitro* experimentations have their limitations, they nonetheless allow a quick and straightforward characterization of proteins in terms of their activity, oligomeric state and ability to interact with other components of the cell. Limitations include where we observe a lack of dimerization *in vitro*, the situation *in vivo* could be very different. For example, dimerization of a protein could be driven by other cellular components and by modification of the protein in question. However, with this in mind, we investigated the properties of a select number of BRAF kinase mutants (L597V, L597V-V600E, D594V, E586K, E586K-V600E and T599W) found in or near to the activation loop (the D^{594}FG motif to Gly 615) of BRAF. We analysed the oligomeric state of the purified kinase domains, their kinase activity and their ability to interact with CDC37. Our results suggest that these mutants were predominantly monomeric *in vitro*, including L597V, which sets it apart from L597R [26]. Our results suggest, that even though the same site (Leu 597) is mutated, the precise mutation, in this case can impose different properties on the purified kinase domain. Furthermore, *in vivo* it was reported that the L597R mutant is sensitive to the BRAF R509H mutation, suggesting that in this context dimerization of the L597V mutant remains a mechanism through which L597V drives carcinogens [23,36].

Another surprising finding is that although E586K and T599W are both monomeric as purified proteins, they both displayed significant amounts of kinase activity. By extension to the results seen for T599W, the oncogenic mutation T599I, might similarly be active as a monomeric protein and further research is required to confirm this. Furthermore, The T599W mutant displayed a weak interaction with CDC37, suggesting that this mutation was able to stabilise the mutant kinase domain in such a way that its interaction with CDC37 was somewhat compromised. Clearly, to understand the details of this change requires structural and biochemical investigations that are beyond the scope of this manuscript. But, nonetheless, it offers an opportunity to further understand the molecular interaction of BRAF and CDC37.

Collectively, our results show unexpected findings for some of the BRAF mutants we studied. In particular the monomeric forms of E586K and T599W displayed kinase activity. While this does not negate their ability to signal as dimers *in vivo*, it does raise the question as to what degree monomeric-kinase-domain activity might play in disease progression for these class II mutants of BRAF, which subsequently raises questions about how to best treat such mutants in the clinic. Additionally, there are observations by other authors that raise similar questions. Noteworthy is the report that L597R was previously shown to cause a strong activation of CRAF [56]. It has also been reported that for some specific class-II BRAF mutants that there are variable and overlapping levels of enriched RAS alterations [30,57], but also with NF1 alterations [31]. However, evidence suggests that specific class-II BRAF alterations have a higher frequency of RAS dependency than class-I mutants, such as V600E. Finally, it has also been observed that for class-II BRAF mutations, a combined BRAF and MEK inhibitor may be a superior approach for treatment [58]. In this scenario, it is possible that the BRAF inhibitor may prevent access to RAS and 14-3-3 by inhibiting its association with the Hsp90-CDC37 complex. In fact, previously, we had shown that the BRAF ATP-competitive inhibitor, vemurafenib, blocked association of BRAF with CDC37 [12]. However, whatever the precise scenario for the activation of specific class-II BRAF mutants is, it is clear that not all class-II mutants are equal. Hence a re-evaluation of whether RAS or simply 14-3-3 alone (or both) oversees the signalling and activation of class-II BRAF mutants and whether active monomeric forms of the kinase also play a role in driving carcinogenesis warrants further investigation.

Author Contributions

Conceptualization, C.P.; methodology, D.M.B., C.P. and X.J.; formal analysis, C.P.; writing—original draft, C.P.; writing—review & editing, C.P., D.M.B. and X.J.; supervision, C.P. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

Not applicable.

Data Availability Statement

Raw data for ITC, SDS-PAGE gel-filtration gels and western blots can be found https://doi.org/10.25377/sussex.29820833.

Conflicts of Interest

Given the role as Editor-in-Cheif, Chrisostomos Prodromou had no involvement in the peer review of this paper and had no access to information regarding its peer-review process. Full responsibility for the editorial process of this paper was delegated to another editor of the journal.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

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