Phosphorylation of RAF Kinase Dimers Drives Conformational Changes that Facilitate Transactivation


Abstract: RAF kinases are key players in the MAPK signaling pathway and are important targets for personalized cancer therapy. RAF dimerization is part of the physiological activation mechanism, together with phosphorylation, and is known to convey resistance to RAF inhibitors. Herein, molecular dynamics simulations are used to show that phosphorylation of a key N-terminal acidic (NtA) motif facilitates RAF dimerization by introducing several interprotomer salt bridges between the αC-helix and charged residues upstream of the NtA motif. Additionally, we show that the R-spine of RAF interacts with a conserved Trp residue in the vicinity of the NtA motif, connecting the active sites of two protomers and thereby modulating the cooperative interactions in the RAF dimer. Our findings provide a first structure-based mechanism for the auto-transactivation of RAF and could be generally applicable to other kinases, opening new pathways for overcoming dimerization-related drug resistance.

RAF kinases connect the Ras GTPase to activation of the MEK-ERK pathway. This pathway regulates many fundamental cellular functions, including cell proliferation, and is dysregulated in approximately 50% of human cancers.¹ This pathway has thus been a key focus in cancer drug development. A recent breakthrough came with the BRAF inhibitor vemurafenib, which achieved high response rates in BRAF-mutated metastatic melanoma.² Interestingly, BRAF inhibition in RAS-mutated tumors induces paradoxical ERK activation and tumor progression owing to the formation of RAF dimers.³ RAF dimerization is also a major mechanism of acquired clinical resistance to RAF inhibitors.⁴ Owing to its important clinical implications, RAF dimerization has attracted enormous interest. RAF homo- and heterodimers show significantly higher kinase activity than monomers, and it has been shown that physiological RAF activation involves dimerization.⁵ Dimer activity remains high even when one protomer (denoted as the activator) is kinase-dead or inhibited, owing to allosteric transactivation of its binding partner (the receiver).⁶ Recent data indicate that the N-terminal acidic (NtA) motif⁷ is essential for the allosteric activation of RAF dimers.⁸ This region is located just upstream of the kinase domain and mediates physiological activation. In RAF1, phosphorylation of the corresponding sequence SSYY (residues 338–341) is induced during RAF1 activation.⁹ In BRAF (residues 446–449, sequence SSDD), the activating site S446 is constitutively phosphorylated and the tyrosines are replaced by negatively charged aspartates. This configuration of the NtA motif primes BRAF for activation, which may explain why single mutations of BRAF, such as V600E in the activation loop, can cause full activation and drive cancer, while RAF1 mutations are rare in cancer.⁹ There is no consensus on which kinase phosphorylates the NtA motif in vivo, since several kinases, including RAF1 itself, have been reported to be able to do this.¹⁰ As shown by mutagenesis studies,¹¹ the NtA motif in the activator is required for transactivation of the receiver in RAF dimers. However, no structural evidence is available to explain this allosteric activation process, since all RAF crystal structures lack the NtA motif.

Based on recently available crystallographic data for RAF (e.g., PDB entries 4E2G¹¹ and 3OMV⁶,¹² we modeled RAF homo- and heterodimers that include the NtA region and correspond to the smallest subset of residues present at the N terminus of the kinase domain in constitutively dimerized, drug resistant splice variants⁹ (Figure 1a). By using atomistic molecular dynamics (MD) simulations of kinase dimers,¹³ we also investigated the role of phosphorylation, which is biochemically well documented but has eluded detailed...
The computational modeling approach and the parameters used are described in the Methods section of the Supporting Information and summarized in Table S1. Phosphorylation of the NtA motif generates several salt bridges that extend and stabilize the binding interface between two BRAF protomers (Figure 1b). Intriguingly, these salt bridges are primarily interprotomer salt bridges, which are formed between the NtA motif and positive residues located either upstream of the NtA motif or at the C-terminal end of the $\alpha_C$-helix, the orientation of which plays an important role in kinase activation.\cite{12a,13}

We note that all of the residues that form interprotomer salt bridges with phosphorylated residues of the NtA motif are conserved in the three RAF isoforms, but not in other kinases (Table S2 in the Supporting Information). Remarkably, the C-terminal end of the $\alpha_C$-helix is neutral in most kinases, whereas in RAFs it carries a +3 charge. Moreover, mutations of these charged residues impair kinase activity\cite{14} and affect RAF homo- and heterodimerization.\cite{16b,14,15}

Since RAF kinases dimerize via the C-terminal end of the $\alpha_C$-helix, the accumulation of six positive charges at the dimerization interface enables interaction with the highly negatively charged NtA motif (specific to RAF), thereby promoting the dimerization. In fact, we estimate that NtA phosphorylation constitutes almost half of the interaction potential energy between the BRAF protomers (Figure 2a and Table S1), predominantly by enhancing electrostatic interactions.\cite{16a}

In particular, we identified two conserved Arg residues, R443 (R336) at the NtA region and R506 (R398) close to the $\alpha_C$-helix in BRAF (RAF1), that participate in the intermolecular salt bridges. R506 (R398) has been reported previously to play a role in dimerization,\cite{14} and here we confirmed the relevance of R443 (R336) experimentally by co-immunoprecipitation, demonstrating a reduced dimerization propensity of R443A mutants (Figure 2b).

Our simulations also reveal that the phosphorylated NtA motif is connected to the active site via the R-spine (Figure 3a). This conserved hydrophobic structure connects four residues from critical sites in the kinase monomer,\cite{16a,17} including the active site. The R-spine is anchored to the $\alpha_F$-
observed that W450 extends the R-spine by forming stacking interactions with the fourth residue (R4, Figure 3a). Although W450 is highly conserved in most kinases (Table S2), the interaction between this Trp and the R4 residue of the R-Spine is not always observed. R4 is aliphatic in about 80% of the kinases, including PKA. In PKA, CDK2, and p38 structures, the R-spine and the corresponding Trp do not interact. Although this Trp is not conserved in EGFR, crystal structures show that its functional equivalent lysine (L680) also interacts with the R-spine. Remarkably, mutation of L680 destabilizes the EGFR dimer in the active complex and impedes kinase activity.[18] We observed that the NtA motif together with W450 extends the dimerization interface, thereby connecting the R-spines and thus the distant active sites of the two protomers. For PKA, aliphatic-to-aromatic mutation of R4 gives normal levels of catalytic activity.[19] This is also the case for BRAF F516A and F516L mutations, which maintain the catalytic activity (Figure 3b) because they preserve the integrity of the R-spine within the monomer. However, we found that these BRAF mutants are unable to transactivate RAF1 in the heterodimer (Figure 3c). These results provide a first structure-based explanation for the transactivation mechanism of RAF dimers following NtA motif phosphorylation.

Importantly, our results indicate phosphorylation-induced large-scale structural changes in RAF dimers, whereas the unphosphorylated dimers remain structurally similar to their starting structures, in agreement with the crystallographic structures. Only when all phosphorylated residues, including the two activation-loop residues near V600, are present in the simulated system, we observe large changes between the universally conserved HRD and DFG motifs (Figure 4). These changes provide insight into the structural flexibility

helix via a hydrogen bond between a carboxylate group at the N-terminal end of the α-helix and the backbone of the HRD motif.[18] An assembled R-spine indicates active kinase conformations, while a broken R-spine correlates with inactive kinase conformations.[16]

Previous experiments showed that mutation of a conserved Trp residue (W450 for BRAF, Table S2) impedes transactivation and impairs the dimerization of BRAF.[17] We
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