Key Oncogenic Ras Mutations Impair Limiting Proton Transfer Rates

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ABSTRACT: Ras-positive cancer constitutes a major challenge for medical treatment. Hot spot residues Gly12, Gly13 and Gln61 constitute the majority of oncogenic mutations which are associated with detrimental clinical prognosis. Here we present a two-step mechanism of GTP hydrolysis of the wild type Ras.GAP complex using QM/MM free energy calculations with the finite-temperature string method. We found that the deprotonation of the catalytic water takes place via the Gln61 as a transient Brønsted base. We obtained reaction profiles for key oncogenic Ras mutants G12D and G12C, reproducing the experimentally observed loss of catalytic activity, and validating our reaction mechanism.

The Ras protein isoforms are essential components of key signaling networks to promote cell proliferation and survival.[1] It is the most frequently mutated protein in all cancer. Ras oncogenes are involved in more than 30% of all human cancer,[2–5] including 98% of pancreatic cancer,[6] 52% of colorectal cancer[7,8] as well as in melanoma[9–11], and lung cancer.[12,13] Additionally, the prognosis for Ras-positive cancer cases is significantly worse than without Ras mutations.[7,11,14–16] Despite more than three decades of extensive research, no effective pharmacological inhibitors of the Ras oncoproteins have reached the clinic, terming Ras proteins as 'undruggable'.[17–19] New therapies are therefore highly sought after.

Ras is a small GTPase, that binds GTP with very high, picomolar affinity (Figure 1).[17] In its GTP-bound form, Ras is active and promotes signaling for cell proliferation. To turn signaling off, [20,21] Ras hydrolyses GTP to GDP with the help of GTPase-activating proteins (GAPs), typically p120GAP or Ras p21.[22,23] GAP completes the environment around the active site (Figure 1A), it contains key conserved motifs, including an arginine finger (Figure 1B)[24] to enable effective catalysis. However, key oncogenic mutations render Ras catalytically inactive, and thus Ras stays in its active signaling, GTP-bound form.

There are three principal isoforms of Ras: KRas, HRas and NRas.[2] The differences between these are mainly related to localization and trafficking of the proteins to reach their signaling partners, while their active sites are identical. Importantly, the most frequent oncogenic mutations correspond to only three active site residues: Gly12, Gly13 and Gln61 (Figure 1C), totaling to over 97% of all Ras mutations.[3] Here we focus on the key oncogenic mutation site Gly12. G12D is overwhelmingly the most frequent Ras mutation, present in half of the Ras positive cancers.[2] We also investigated G12C as there are novel promising covalent inhibitors (AMG510 and MRTX849) that react with the cysteine sidechain and have already reached phase 3 studies. [25–29]

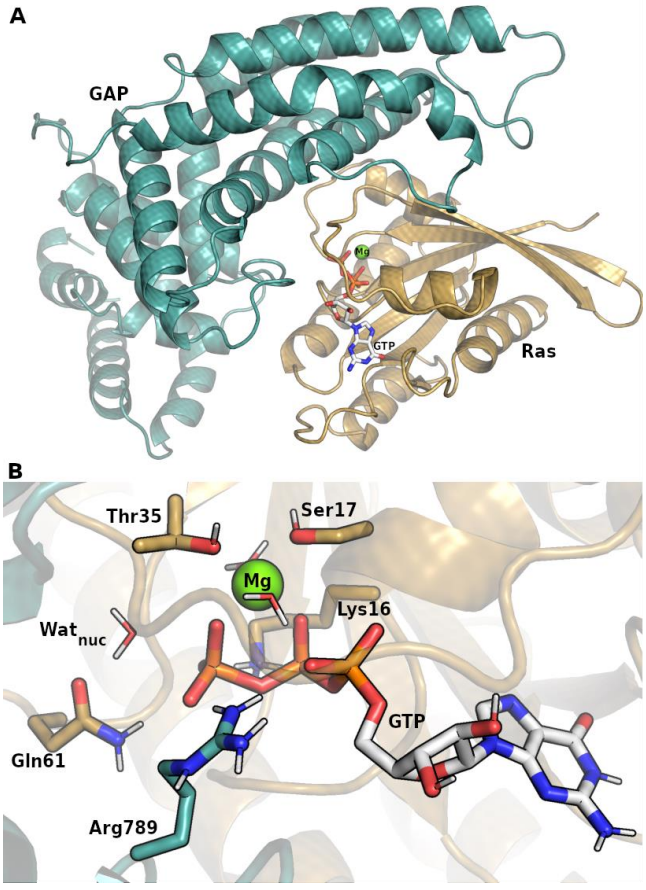

Figure 1. **A:** Ras (gold cartoon)-GAP (blue cartoon) model based on PDB ID 1WQ1. **B:** GTP (white sticks) alongside with Mg^{2+}-coordinating residues. Arginine finger (blue sticks) from p120GAP coordinates the GTP.

Experimentally, Ras structures are well characterized, and transition state (TS) analogues are available in Ras.GAP bound complexes.[30] We used the Ras.p120GAP complex (PDB ID 1WQ1) as the starting structure for our simulations (Supporting Information section I).[31]

The active site of Ras and the main associative phosphate cleavage reaction is well established (Figure 2A). An essential Mg^{2+} ion coordinates the β- and γ-phosphates,[32] Ser17, Thr35 of the RAS effector lobe and two water molecules.[33] The nucleophilic water molecule is positioned near the γ-phosphate via H-bonding to Gln61:Oε and the Gly60 backbone. The important arginine finger, Arg789 of the GAP coordinates the GTP.

The catalytic mechanism, however, still leaves many questions unanswered. The main controversy involves the proton transfer mechanism of the GTP hydrolysis reaction.[34,35] Upon hydrolysis, the nucleophilic water gets deprotonated while one of the oxygens of the formed inorganic (dihydrogen)phosphate (P_i) gets protonated. Potential mechanisms were proposed to be (i) a direct transfer (substrate assisted or 1 water, 1W mechanism, Figure *2*B), (ii) via an additional water molecule (solvent assisted or 2 water, 2W mechanism, Figure *2*C), or (iii) catalyzed by a basic protein residue (general base assisted, Figure *2*D).[34]

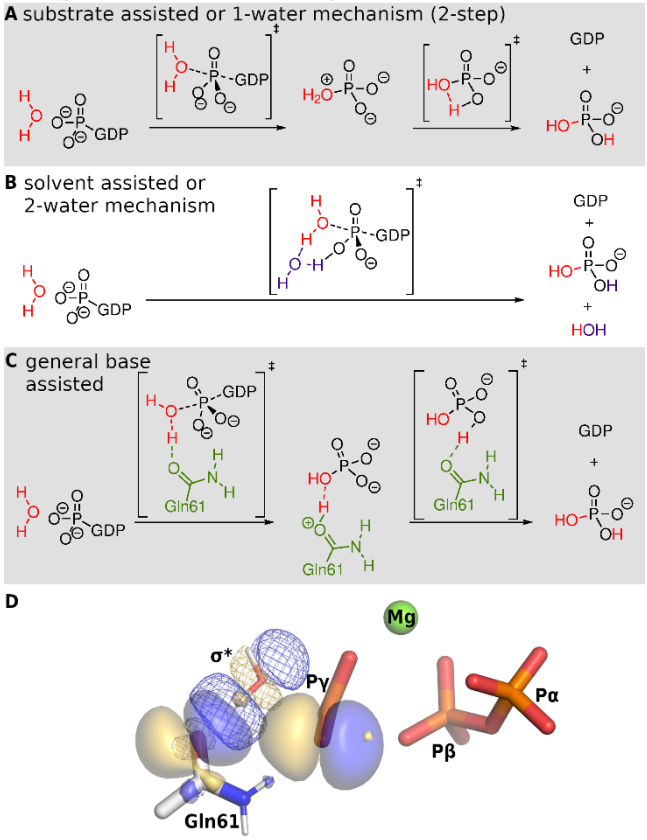

Figure 2. **A-C:** Proton transfer alternatives during GTP hydrolysis. **D:** Natural bonding orbitals during the phosphate cleavage. Solid surfaces represent occupied NBOs (lone pairs), meshes depict the virtual antibonding orbital of the Wat_{nuc} O-H bond. The electron donation from the axial direction by Oε of Gln61is more favorable than the donation from the phosphate oxygen.

Despite multiple studies proposing reaction mechanisms for wild type (WT) Ras, very little is known about how detrimental changes in enzyme activity are induced by oncogenic mutations. Experimental kinetic measurements are nevertheless widely available for WT and mutant Ras proteins,[12,22,36] pointing to the loss of catalytic activity due to the impaired rate of hydrolysis. Computational studies elaborated on the changes in the reactant state (RS, Figure 3A) Ras.GTP complex structures upon Gly12, and Gln61 mutations,[37–43] including in-depth analysis of the changes in atomic charges and the polarization of the active site before the reaction.[44] However, calculations to evaluate the influence of the important oncogenic changes on the reaction mechanism are missing.

To assess the structural changes caused by the key oncogenic mutations of Gly12, G12C and G12D, we analyzed classical molecular dynamics (MD) trajectories (Supporting Information section II). In general, the Cys12 substitution causes a less disruption in the active site conformations, while the Asp12 substitution induces more notable changes, such as weakening the interaction of the GTP with the Switch I loop (Table S2-3). Importantly, both mutations affect the contact with Gln61, and the interactions with the side chain are about 50% present during the simulations, while with Gly12 such interactions are absent. Given the essential role of Gln61 in the hydrolysis, this interaction is likely to contribute to the diminishing activity. The stabilizing role of Gln61 in the H-bonding pattern in the RS was previously also highlighted.[45] Accordingly, G12C and G12D mutations were found to induce conformational changes in Gln61.[37] Re-arrangements of water molecules were observed at the active site, consistently with our MD simulations. The disturbance of the water distribution was also observed in many Gln61 mutants.[46] Nevertheless, no major structural changes were otherwise identified in the active site. Therefore, these changes alone may not account for the major loss of activity in the Gly12 mutants.

To reveal how these key oncogenic mutations act on the catalytic pathway, we first explored the WT Ras.GAP reaction mechanism, including the proton transfer steps using QM/MM free energy calculations (Supporting Information section IV). We found that the substrate assisted transfer (1W) to the phosphate (Figure *2*B) has a large barrier (Figure S2) and it is likely unfeasible due to the orbital orientation of the breaking bond. Figure 3B depicts two lone pair Natural Bonding Orbitals (NBOs) that may donate electron density towards the unoccupied O-H antibonding orbital of the Wat_{nuc} to demonstrate the significant advantage of the orientation provided by Gln61. The perturbation of the Gln61:Oε lone pair is two orders of magnitude higher than that of the lone pair of the O3γ (Table S5). We therefore included additional water molecules to facilitate this proton transfer (Figure *2*C), however, these attempts also produced a high barrier (Figure S3). The importance of Gln61 was recognized by early studies,[47,48] by activating the Wat_{nuc}. Initially, we used constrained QM/MM minimizations to explore the mechanism to form the phosphate product by tautomerizing Gln61 into an imide, suggested by Warshel et al.[49] and Nemukhin et al.[50] Our attempts to establish an intermediate with the imide form of Gln61 failed and the Nε regained the proton from the phosphate.[51] Instead, we obtained the lowest barrier energy minimized path via a transient proton transfer to the key Gln61 residue via Gln61:Oε (Figure *2*D). In our simulations, the rate-determining step is the protonation of the inorganic phosphate by the transient $GlnH^+$. A similar mechanism was proposed recently by Nemukhin et al for the catalytic

mechanism of Ran GTPase,[51,52] and was also listed as one of the possible options for the Rho GTPase mechanism by Blackburn et al.[53] Previous calculations based on the PM3 semiempirical method suggested that the Gln61 is not basic enough,[54] which underlines the need for high level QM methodology. Gln61 was nevertheless suggested to serve as a base in very early studies,[55] although we find that the proton transfer is tightly coupled to the phosphate cleavage and does not take place *a priori* as a separate step.[55]

The five stationary points of our proposed mechanism are depicted in Figure 3. The first transitions state (TS1) corresponds to the nucleophilic substitution on the phosphorus and the proton transfer from Wat_{nuc} to the Gln61 (Figure 3C). The obtained intermediate (Figure 3D), characterized by the protonated Gln61, is in strong H-bonding interaction with the newly formed inorganic phosphate. This interaction is being broken during the second, rate-limiting transition state (TS2, Figure 3E), whereby the phosphate rotates to enable the proton transfer from the Oε of the Gln61. In the direct product complex (PS, Figure 3F), the P_i remains in coordination with the Mg^{2+}.

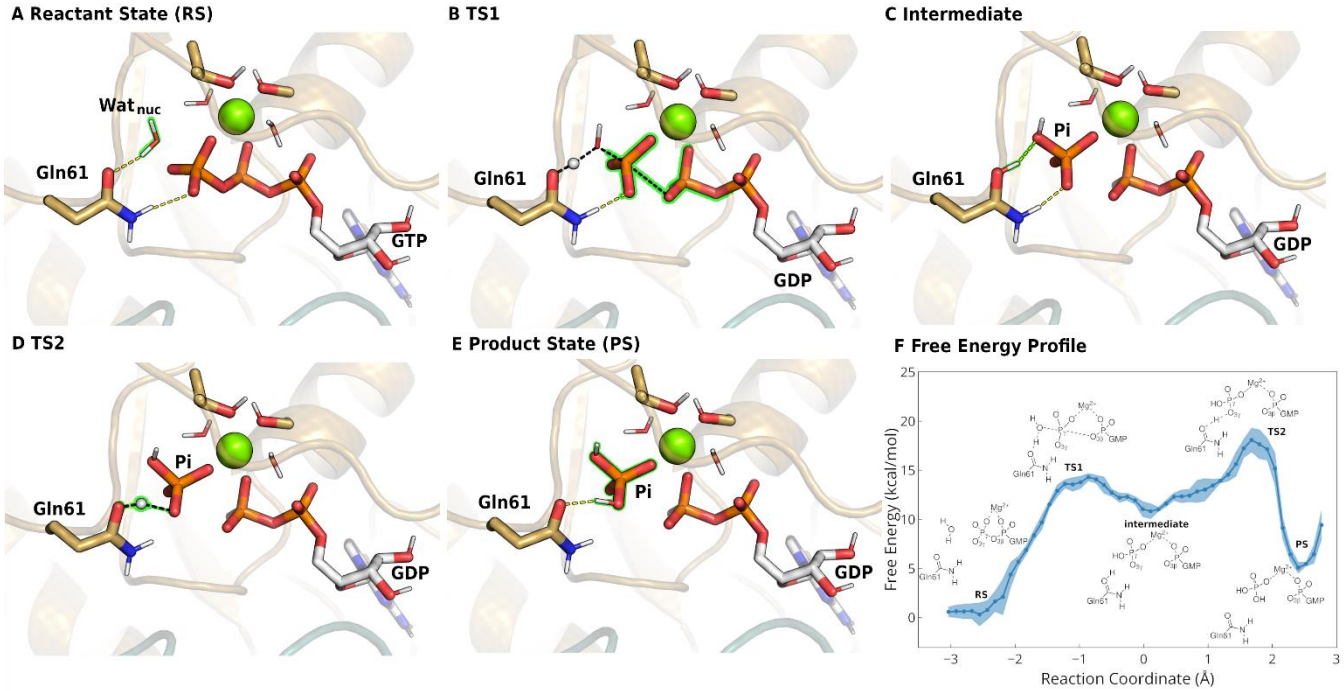

Figure 3. Stationary points along the wild type Ras.GAP GTP hydrolysis. Breaking and forming bonds (black dashes), hydrogen bonds (yellow dashes) are depicted. **A:** Reactant state. **B**: First transitions state. **C:** Intermediate with protonated Gln61. **D:** Second transition state. **E:** Product state of a bound GDP+P_i. For clarity, non-polar hydrogens are omitted. **F:** Free energy reaction profile from string calculations projected along reaction coordinate, as defined in the Supporting Information section VII. Shades depict the estimated variation of the profile along the energy axis. Stationary structures are drawn schematically.

The optimized reaction profile was used as the starting point for the finite-temperature string method (Supporting Information section VII). The free energy profile is reconstructed using WHAM[56] and is depicted, along with the estimated uncertainty, in **Error! Reference source not found.**. The overall barrier corresponds to the second, rate-determining step is 18.1±1.6 kcal/mol, in good agreement with experimental rates (Table 1).

Nevertheless, despite that the current mechanism seems highly likely, we cannot exclude larger structural changes that might accompany, or prelude, the second proton transfer. This is also possible, considering available structural data, as GDP-bound Ras has a distinct switch I-II domain conformation,[57] and such a conformational change must take place after the cleavage of the gamma phosphate. However, the current QM/MM-based methods would not be able to capture such significant structural rearrangements, even if the timescale is fast, and future work will be needed to evaluate this mechanism.

Subsequently, using our WT mechanism as the starting point, we also investigated the reaction paths for the G12D and G12C replacements. Reaction barriers from constrained QM/MM minimizations along the path (Figure 4, orange and red, respectively) are in good agreement with experimental rates (Table 1).

Table 1. Computational and experimental activation barriers of GTP hydrolysis catalyzed by Ras.GAP.

	WT	G12C	G12D
calculations	**18.1**		
	22.5	*24.3*	*27.1*
experiments	16.4[a]	23.1	24.3
	20.1[b]	23.4	22.5
	21.4[c]	-	24.4

Bold: free energy based on string calculations; Italic: potential energies obtained from constrained optimizations. Experimental barriers were calculated from rates assuming first-order kinetics. [a]Wey et al.[58] [b]Hunter et al. [36] [c]Johnson et al.[59] All energy values are in kcal/mol.

G12C presents a smaller change of 1.8 kcal/mol in the activation barrier of the Ras.GAP reaction in accordance with the smaller structural changes observed during the MD simulations. It only increases the barrier of the second step, required to complete the proton transfer to the inorganic phosphate. On the other hand, the G12D barrier is higher than the WT for both steps, increasing the barrier by 4.6 kcal/mol. Moreover, the second transition state position along the reaction coordinate is shifted towards the intermediate. This is due to the destabilization of the intermediate as the proton transfers to the Gln61 earlier than in the other cases (Figure S4). Interestingly, the proton transfer to Gln61 occurs earlier in the reaction path (Figure S4) The comparison of the NBO charges reveals that in the first reaction step the electron density (as measured by the cumulative charge) at the attacking water is slightly reduced by the G12D mutation (+0.035). This lowers its nucleophilicity and is thereby a possible explanation for the observed barrier increase. In the case of the G12C mutation, this change is significantly smaller (+0.008) and the barrier does not change compared to the WT (Table S8). This is consistent with experimental rates observed by Wey et al, whereby a larger change in the rates is observed for G12D.[58]

Our general base assisted mechanism is also supported by experimental findings that the Q61E mutant Ras has an increased intrinsic GTPase activity.[54,60] In other phosphatases, a stronger Brønsted base is often used. For example, the GTPases hGBP1[61] and FeoB,[62] as well as the ATP dependent myosin motor domain [63] use a glutamate as a base, accessed through a proton relay. Analogous roles for sidechain-assisted proton transfer also involves aspartate (e.g., for dUTPase[64]) or histidine residues (for RNase H, RNase T or RuvC)[65] in other phosphate cleaving enzymes. Nevertheless, the identification of the base is often a challenge for mechanistic studies.

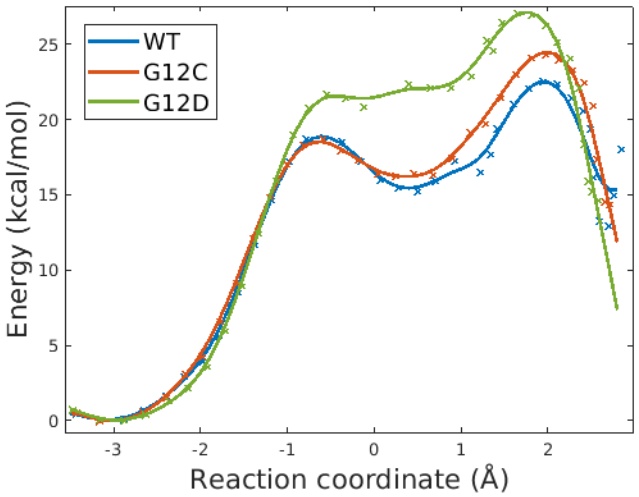

Figure 4. QM/MM energy from constrained minimizations of the WT (blue), G12C (red), and G12D (green) Ras using the reaction coordinate, as defined in the Supporting Information section VII.

In conclusion, we present a detailed mechanism for Ras.GAP catalyzed reaction using QM/MM free energy calculations. Importantly, the obtained mechanism also allows us to compare reaction rates for two key oncogenic mutations: G12C and G12D. The agreement observed with experimental rates validates the detailed proton transfer steps that involve the crucial Gln61 residue as proton acceptor. This mechanism opens up a starting point for computational screening to develop small molecule binders that, instead of inhibiting the enzyme reaction, restore the GTPase activity of oncogenic Ras and turn aberrant signaling off.

ASSOCIATED CONTENT

Computational details, alternative mechanism and NBO analysis (PDF)

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All authors have given approval to the final version of the manuscript.

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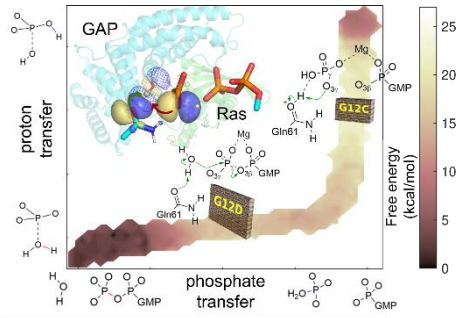
GAP
Ras
Mg
GMP
G12C
G12D
Gln61
proton
transfer
phosphate
transfer
Free energy
(kcal/mol)
25
20
15
10
5
0