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Conformational and Dynamical Effects of Tyr32 Phosphorylation in K-Ras: Molecular Dynamics Simulation and Markov State Models Analysis

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Abstract

Phosphorylation of tyrosine 32 in K-Ras has been shown to influence function by disrupting the GTPase cycle. To shed light on the underlying mechanism and atomic basis of this process, we carried out a comparative investigation of the oncogenic G12D K-Ras mutant and its phosphorylated variant (pTyr32) using all-atom molecular dynamics simulations and Markov state models. We show that, despite sharing a number of common features, G12D and pTyr32-G12D K-Ras exhibit some distinct conformational states and fluctuations. In addition to notable differences in conformation and dynamics of residues surrounding the GTP binding site, nonlocal changes were observed at a number of loops. Switch I is more flexible in pTyr32-G12D K-Ras while switch II is more flexible in G12D K-Ras. We also used time-lagged independent component analysis and k-means clustering to identify five metastable states for each system. We utilized transition path theory to calculate the transition probabilities for each state to build a Markov state model for each system. These models and other close inspections suggest that the phosphorylation of Tyr32 strongly affects protein dynamics and the active site conformation, especially with regards to the canonical switch conformations and dynamics.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.jpcb.9b05768](https://doi.org/10.1021/acs.jpcb.9b05768). Figures showing secondary structure profiles, dynamic cross correlation maps, the Chapman–Kolmogorov MSM validation test, and RMSD matrices of selected regions computed from MSM metastable states trajectories (PDF)

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INTRODUCTION

The Ras protein family represents one of the best characterized and ubiquitously expressed group of small GTPases. Human Ras proteins consist of three isoforms: H-, K-, and N-Ras.¹ Of particular interest is the K-Ras isoform, which is anchored to the intracellular leaflet of the plasma membrane via a polybasic domain with a farnesylated C-terminus.^{2,3} The K-Ras structure features two main components: the catalytic domain (Figure 1B, amino acids 1–166) and the membrane-targeting hyper variable region (HVR, amino acids 167–185 + farnesyl anchor). The membrane-anchoring region is not conserved, and it is characterized by notable sequence differences among the Ras isoforms (Figure 1A). The highly conserved catalytic domain of K-Ras interacts with effectors and exchange factors via conformational changes at its flexible canonical switches: switch 1 (SI, residues 25–40) and switch 2 (SII, residues 60–75). Similar to other Ras isoforms, K-Ras regulates crucial signaling pathways controlling cell growth, proliferation, and differentiation.^{4–6} Consequently, a significantly large number of all human cancers are associated with K-Ras mutations, making it an increasingly popular anticancer drug target.^{7,8} However, drug design efforts have been impeded by our limited understanding of the complex interplay among competing binding processes.

Many computational and experimental studies have shown that the complex biological functions of K-Ras depend upon a set of conformational changes accompanying its binding to membranes and partner proteins. Conformational changes also occur during the hydrolysis of GTP to GDP, a process that switches Ras from the active state to the inactive state.^{9,10} Similarly, conformational changes associated with membrane binding, dimerization,^{11,12} oligomerization,¹³ and mutation¹⁴ are crucial for K-Ras function. The phosphorylation of specific amino acids is another process that has significant effects on protein conformation and function. Phosphorylation alters the local chemical environment in the modified and adjacent residues.^{15,16} Presumably through a similar mechanism, phosphorylation and dephosphorylation mediated by Src and SHP2 protein tyrosine phosphatase (PTP)¹⁷ have been shown to modulate Ras activity. Phosphorylation induced by Src regulates the GTPase cycle by impairing Raf binding.¹⁸ In contrast, dephosphorylation induced by SHP2 negatively impacts the GTPase cycle by enhancing Raf binding. Therefore, any disruption to the balance of these processes may lead to adverse effects including cancers. Recent biophysical experiments have suggested that phosphorylation of Tyr32 of K-Ras attenuates its sensitivity to GAP and GEF, enhances intrinsic nucleotide exchange, impairs intrinsic GTP hydrolysis, and enhances binding to effector Raf.¹⁹ Tyr32 phosphorylation is thought to alter SI and SII conformations due to electrostatic repulsion against the negatively charged Asp38 and Asp57 in the nucleotide-binding site.²⁰

Although the aforementioned studies have revealed the importance of Tyr32 phosphorylation, its effect on K-Ras structure and dynamics is still not fully understood. To overcome experimental limitations and gain further insights into the mechanism of conformational changes due to Tyr32 phosphorylation, in this work we investigated the underlying structural and dynamical changes using molecular dynamics simulations of the GTP-bound catalytic domain of G12D K-Ras and its phosphorylated variant pTyr32-G12D. We identified a number of metastable conformational states and the kinetic network of transitions between them using Markov state model (MSM).

MATERIALS AND METHODS

Initial Structures and System Preparation.

The initial structure of G12D K-Ras was obtained from PDB entry 4DSO which is also used to prepare the starting structure for the phosphorylated variant by mutating Tyr32 to pTyr32. In both systems, GSP was swapped with GTP, and all other molecules found in the X-ray crystal structure were removed except for crystal water molecules and Mg²⁺ ions. Protonation states of N-terminus (protonated), C-terminus (deprotonated), cationic residues (protonated), and anionic residues (deprotonated) were determined based on a neutral pH local environment. The resulting structures were solvated in a pre-equilibrated cubic solvent box containing TIP3P water molecules. Solvent padding was at least 10 Å so that the protein does not interact with its periodic images. Finally, sodium and chloride ions were added to make each system electrically neutral and achieve a physiological ionic strength of 150 mM.

Molecular Dynamics Simulations

Periodic boundary conditions in all directions were utilized to reduce finite system size effects. Short-range nonbonded interactions were smoothly switched off between 10 and 12 Å. Nonbonded pair lists were built using a 14 Å cutoff and updated every 20 fs. Long-range electrostatic interactions were computed using the particle mesh Ewald (PME) method²¹ with fast Fourier transform grid density of approximately 1.0 grid/Å. The energy of both systems was minimized using 5000 steps of conjugate gradient to remove any local atomic clashes. Subsequently, the systems were steadily heated at a pace of 0.2 K/fs while keeping the C α and GTP heavy atoms restrained by a harmonic restraint of force constant $k = 4.0$ kcal mol⁻¹ Å⁻², and then equilibrated with k gradually reduced to zero at a rate of 0.01 kcal mol⁻¹ Å⁻² ps⁻¹. An integration time step of 2 fs was used along with the SHAKE²² algorithm to constrain covalent bonds involving hydrogen atoms. Both systems were equilibrated for 10 ns in the isothermal–isobaric (*NPT*) ensemble at the physiologic temperature of 310 K which was maintained using Langevin dynamics with a damping coefficient of 10 ps⁻¹. The Nose–Hoover Langevin piston method was utilized with a piston period of 200 fs and decay time interval of 100 fs to maintain a constant reference pressure of 1.0 atm. For the production runs, each system was simulated for 500 ns under the same input parameters and *NPT* conditions. All simulations were carried out using NAMD2.11²³ and the all-atom empirical force field CHARMM27 with CMAP dihedral angle correction.²⁴

RESULTS AND DISCUSSION

Effect of Tyr32 Phosphorylation on the Structure and Dynamics of SI, SII, and Helix $\alpha 3$ Regions.

Significant differences are observed between G12D K-Ras and its phosphorylated counterpart, particularly in terms of conformational flexibilities of few regions. This can be seen even from the root-mean-square deviations (RMSD) shown in Figure 2. Overall, the structures are very stable for both mutants with backbone RMSDs from the initial X-ray structure remaining below 1.5 Å except at the switch regions, especially in pTyr32-G12D. In addition, Tyr32 phosphorylation of G12D K-Ras does not induce severe structural changes as evident from the secondary structure content. Figure S1 indicates that there are no noteworthy differences in the secondary structure profiles from both simulations. C α atom root-mean square fluctuations (RMSFs) per residue of both proteins (Figure 3) show that SI and helix $\alpha 3$ are more flexible in pTyr32-G12D (average RMSF of SI residues is 1.53 Å versus 1.12 Å in the unphosphorylated system), while SII is less flexible in pTyr32-G12D (average RMSF of SII residues is 0.79 Å versus 0.97 Å). The decreased flexibility in SII could affect binding and activation of effectors by affecting the dynamic interactions involving residues outside the effector binding region.^{25,26}

Visual inspection of the trajectories showed that helix $\alpha 3$ is shifted toward switch II in G12D due to interactions that do not exist in pTyr32-G12D. These include transient hydrogen bonds: Arg68:NH1-Tyr96:OH, Arg73:N-Val103:HZ1 and Gly75:O-Lys104:HZ2. Not only does this explain the differential flexibility of SII and helix $\alpha 3$ between the two proteins but also it suggests differential communications between lobe 1 (residues 1–86) and lobe 2 (residues 87–171). It is also consistent with the suggested functional significance of

Tyr32 phosphorylation given the well-documented correlated motions between helix $\alpha 2$ and helix $\alpha 3$ in GTP-bound Ras.²⁷

The GTP binding pocket of K-Ras is overall positively charged, which helps to stabilize the negatively charged phosphate groups of GTP. In addition, Tyr32 plays a role in coordinating a water molecule adjacent to the γ -phosphate of GTP and modulating intrinsic hydrolysis.^{28,29} Tyr32's motion is correlated with that of the four negatively charged adjacent residues: Glu31, Asp33, Glu37, and Asp38. These residues play a critical role in effector binding. Insertion of the additional negative charge on Tyr32 changes the local charge distribution and alters the local interaction network including within the switch regions, the GTP binding pocket and beyond in spatially distant regions. The latter is substantiated from by dynamic cross correlation maps displayed in Figure S2, which show distinct differences in the correlated motion patterns in the aforementioned regions and other regions. Importantly, pTyr32-G12D exhibits reduced correlations among SII, helix $\alpha 3$ (residues 87–104), β strand $\beta 1$ (residues 2–9), P-loop (residues 10–17), β strand $\beta 3$ (residues 49–57) and β strand $\beta 4$ (residues 77–83). It also shows anticorrelations between SI and helix $\alpha 1$ (residues 16–24). Phosphorylation of Tyr32 induces an electrostatic repulsion between the negatively charged carboxyl groups of the two proximal residues Asp38 and Asp57, as can be seen in Figure 4. This is in agreement with the mechanism proposed by Bunda and co-workers.²⁰

Figure 5 shows that the Tyr32-induced reorganization is coupled with the binding of an Na^+ ion to $\text{GTP:O2}'$ as well as Asp30, Glu31, and Asp33. This Na^+ ion stabilizes the motion of Tyr32 side chain and helps it to form a hydrogen bond with Thr35. These conformational changes lead to an alteration in the SI architecture and by extension interactions to effectors.^{30,31} It is also consistent with observations from previous experiments.²⁰ In particular, it has been shown that K-Ras adopts two distinct conformational states that are linked to its activity:^{32,33} an inactive state 1 and an active state 2 conformations. State 2 is characterized by interactions of Thr35 and Gly60 with GTP. Within our simulation time scale, we did not observe conformational transitions between the active and inactivate states. Both mutations mostly remained close to the active state 2 configuration, with the $\text{G60:NGTP:O}\gamma 2$ and $\text{T35:O}\eta\text{-GTP:O}\gamma 3$ distances being 3.1 ± 0.3 and 2.8 ± 0.1 Å. Nonetheless, each protein adopted a different Tyr32 conformation as shown in Figure 6 using the dihedral angle χ^1 ($\text{N-C}\alpha\text{-C}\beta\text{-C}\gamma$) of Tyr32. Clearly, phosphorylation affects Tyr32 side chain orientation. This change in Tyr32 side chain orientation contributes to the changes in the SI dynamics and thus likely have functional significance, especially since Tyr32 undergoes a major reorientation during nucleotide exchange.^{25,34}

To identify the most significant large-scale motions and gain better insights into how the phosphorylation of Tyr32 might affect global protein dynamics, we employed a principal component analysis (PCA) on the concatenated trajectory of both systems using Cartesian coordinates of $\text{C}\alpha$ atoms of residues Asp12, Tyr32, Pro34, Ile36, Gly48, Leu56, Ala59, Glu63, Ala66, Met67, Thr74, Asp105, Asp108, Ser122, Asp126, Gly138, Thr148, and Asp153. Figure 7 shows significant differences between the mutants in terms of the large-scale motions characterized by the first two principal components. Specifically, the phosphorylated variant sampled a larger conformational space than the unphosphorylated

one, indicating that the former is more dynamic than the latter. The differences along PC1 are mainly due to larger fluctuations in the SI of the phosphorylated variant.

Building Markov State Models and Their Validation

To explore the long-lived conformational dynamics for each system and weed out local fluctuations due to thermal motion, we built Markov state models (MSM)^{35,36} using the PyEMMA software package version 2.³⁷ Multiple definitions of microstates were tested. We found that the distances among C α coordinates of residues Asp12, Tyr32, Pro34, Ile36, Gly48, Leu56, Ala59, Glu63, Ala66, Met67, Thr74, Asp105, Asp108, Ser122, Asp126, Gly138, Thr148, and Asp153 are sufficient to resolve the conformational changes observed in the two trajectories. These residues are highly dynamic in nature as can be seen in the RMSF plot (Figure 3), and some of them are located at the effector binding surface. Time-lagged independent component analysis (TICA)³⁶ with a 1 ns lag-time was used to differentiate between slowly equilibrating populations of the input coordinates (Figure 8), and subsequent dimension reduction was achieved by projecting onto the slowest TICA components. K-means clustering was used to get a set of 100 microstates represented by cluster centers. The resulting discretized trajectories were used to construct the Bayesian MSM using a 2 ns lag-time for which the system is considered Markovian (i.e., implied time scales reached a plateau and became independent of the lag time itself, see Figure 9). Spectral analysis of time scale separations shows that the largest time scale separation is between the first and the second relaxation time scales for G12D, and the third and fourth relaxation time scales for pTyr32-G12D, respectively (Figure 10). This suggests that retaining four relaxation times or five metastable states is sufficient to coarse-grain the dynamics of both systems. Thus, the microstates were grouped into five metastable states using the Perron-cluster cluster analysis (PCCA++) method.³⁸

The free energy of each metastable state was computed as a function of the two slowest independent components by comparing the probabilities of its constituent microstates. The Chapman–Kolmogorov test³⁵ was employed to further validate the reliability of the five metastable state Markov state models by comparing the predicted residence probability of each microstate obtained from MSMs with those directly computed from MD simulations. As shown in Figure S3, the probability predicted from MSMs for a given metastable state has small deviations from the histogram counts from MD simulations. Finally, transition path theory^{39,40} was utilized to compute the transition path fluxes among metastable states using the forward committer probabilities because MSM here is reversible.

Structures and Dynamics of Metastable States.

Both MSMs identified five metastable states using the Perron-cluster cluster analysis (PCCA++). The population of each cluster represents closely related conformational states (Figure 11, parts A and B). The free energies for each metastable state (S_i) can be computed from its stationary MSM probability π using

$$\Delta G(S_i) = -k_B T \ln \sum_{j \in S_i} \pi_j$$

where π_j is the MSM stationary weight of the j th microstate (Table 1). The conformational changes between two different conformational states are relatively slow. The slowest MSM time scale of pTyr32-G12D K-Ras is about 220 ns, but it is faster for G12D K-Ras at 55 ns. This suggests that the simulation time is not enough to detect all possible transitions between metastable states for pTyr32-G12D K-Ras. Observing enough number of transitions may require simulations in the order of multiple microseconds. Similarly, it is difficult to observe these dynamic metastable states in static crystal structures due to the fast dynamics of the switch regions.

Our MSM results suggest that both mutations display different metastable state distributions that can be visually distinguished as free energy minima. The phosphorylated G12D K-Ras has one highly populated metastable state with an occupancy probability of 63%, three states with occupancy probabilities of 10% to 14% and one least populated state with an occupancy probability of 3%. G12D K-Ras has one high populated metastable state with an occupancy probability of 39%, three states with occupancy probabilities of 18%–21% and one state with a 1% occupancy. The free energy profiles (Figure 11, parts C and D) indicate that more intermediate conformations among these states were sampled, especially in G12D. In order to gain insight into the mechanisms of conformational transitions in each mutant, we used the transition path theory and obtained detailed transition pathways among the metastable states. Figure 12 shows the structure of each of the metastable states for each system. Differences in MSM metastable state conformation and dynamics of the systems are in agreement with the observed effect of Tyr32 phosphorylation on the switch regions indicated by the RMSD matrix (Figure S4 and S5). For pTyr32-G12D K-Ras, the switch regions exhibit distinct behaviors. In particular, SI shows remarkable conformational differences across all metastable states, but SII does not. State 1 and state 2 have similar conformations and the Tyr32 side chain orientation does not show notable differences. Similarly, comparison of state 4 and state 5 suggests that conformations of both states are closely related with no notable differences in Tyr32 side chain orientation. This suggests that state 3 is an intermediate state. It also indicates that states 1 and 2 and states 4 and 5 exhibit clear conformational differences especially in the SI region. Most notable is the change in side chain orientation of Tyr32, which is a clear measure for delineating the metastable states of pTyr32-G12D K-Ras. As for G12D K-Ras, the SI states I, II, and III have similar conformations with a relatively stable Tyr32 orientation. Likewise, states IV and V exhibit similar SI conformations with a somewhat dynamic Tyr32 orientation. The dynamical and conformational differences in the metastable states of both mutants in the SI region suggests that each metastable state may have a specific role in binding to partner proteins and thereby pathway activation. In particular, Tyr32 phosphorylation may affect Ras-GAP interactions, as a previous study revealed that mutation of negatively charged residues in the SI region affects the electrostatic interaction between Ras and GAP and modulates the binding affinity.

41

Figure 12 shows the transition network of the highest flux pathways among the metastable states of each protein, as obtained from TPT. For pTyr32-G12D, the maximum flux pathway from the initial metastable state 1 to the final metastable state 5 is $1 \rightarrow 2 \rightarrow 4 \rightarrow 5$ and accounts for 42% of the total flux. Other major transition paths with significant fractional flux are listed in Table 2. Similarly, the maximum flux pathway from the initial metastable state I to the final metastable state V of G12D is $I \rightarrow II \rightarrow III \rightarrow V$, which accounts for 55% of the total flux. It is worth noting that system dynamics and the transition rates computed here might be susceptible to the choice of the damping coefficient value in the Langevin dynamics thermostat.⁴² However, the extent of the sensitivity of these rate values to the choice of the thermostat and its parameters is unknown for our system.

CONCLUSIONS

In this study, we identified the structural and dynamic differences that might aid in understanding the underlying mechanism for the observed functional differences between G12D K-Ras and its pTyr32 counterpart. To compare conformational and dynamical differences between the two proteins, we conducted a 500 ns unbiased MD simulation and built Markov state models for each system. Because the changes in protein dynamics are subtle and difficult to quantify, we analyzed conformational and dynamical differences using RMSF, RMSD, PCA, and the dihedral angle of Tyr32 side chain. Our results show that G12D and pTyr32-G12D K-Ras show both shared and unique conformations and fluctuations that could have implications for differential GTPase activity and effector interaction. Significant differences in conformation and dynamics were observed around the GTP binding site, but there were also distinct differences at some distal loops. SI showed more flexibility in pTyr32-G12D K-Ras while SII is more flexible in G12D K-Ras. The simulations also revealed an interaction of a sodium ion with the GTP and neighboring residues in the phosphorylated mutant. The ion coordinates interaction among residues 30, 31, and 33, which facilitates the reorientation and displacement of Tyr32 toward the γ -phosphate position. Finally, MSMs confirmed the effects of Tyr32 phosphorylation on dynamics of the switch regions, and revealed the role of Tyr32 in behavior of the metastable states of each mutant. These results suggest a direct role of Tyr32 dynamics and orientation on GTP hydrolysis and effector binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

G12D K-Ras sequence and structure. (A) Sequence of full length G12D K-Ras protein including the catalytic domain (amino acids 1–166) and hyper variable region (HVR, amino acids 167–189). Switch 1 (SI, amino acids 25–40), switch 2 (SII, amino acids 60–75), residues 12 and 32 are highlighted by red, blue, purple, and green, respectively. (B) G12D K-Ras catalytic domain structure shown in cartoon representation. The location of the mutations at position 12 and 32 are highlighted by purple and green spheres, respectively. SI and SII regions are highlighted in red and blue, respectively.

Figure 2.

Time evolution of root-mean square deviation (RMSD). Backbone RMSD of the entire catalytic domain structure (top), SI region (middle) and SII region (bottom) from the initial X-ray structure for both G12D K-Ras (left) and its phosphorylated counterpart (right). RMSDs were computed after structural alignment excluding the flexible switch regions. Gray lines represent data sampled every 100 ps, and thick black lines represent 10 ns running averages.

Figure 3.
Ca root-mean-square fluctuations (RMSFs). RMSFs were computed after alignment excluding SI and SII regions for both G12D K-Ras (black) and its phosphorylated variant (red). SI and SII are highlighted by cyan and purple shadows, respectively.

Figure 4.

Time evolution of the distance between the C α atoms of Asp38 and Asp57 in G12D K-Ras (black) and its phosphorylated variant (red).

Figure 5. Long-residence sodium-ion binding sites. A snapshot of pTyr32-G12D K-Ras showing a sodium ion interacting with GTP and SI.

Figure 6. Probability of the dihedral angle χ_1 (N-C α -C β -C γ) of Tyr32 for G12D K-Ras (black) and its phosphorylated variant (red).

Figure 7.
Global conformational dynamics of G12D K-Ras and its phosphorylated counterpart.
Projection of simulated conformers into the first and the second principal components for
G12D K-Ras (black) and its phosphorylated counterpart (red).

Figure 8.

Projection of each trajectory onto the two largest TICA components. Gray lines represent data sampled every 100 ps while thick black lines represent 10 ns running averages.

Figure 9.

Validation of the Markov state model. Implied relaxation time scales for the first six eigenvalues calculated from the transition matrix at different lag times. All relaxation time scales become approximately constant beyond the used lag-time of 2 ns to construct MSMs for (A) G12D and (B) pTyr32-G12D.

Figure 10.

Spectral analysis of time scale separation. The spectral analysis revealed that the largest time scale separation is between the first and the second relaxation time scales for G12D, as well as the third and fourth relaxation time scales for pTyr32-G12D.

Figure 11.

Free energy landscape and five metastable states grouped from microstates. Each trajectory was clustered into microstates by assigning its frames to 100 cluster centers using the k -means clustering. The microstates were then grouped by the PCCA++ method into five metastable states. (A) pTyr32-G12D K-Ras with metastable states colored as 1 (blue), 2 (gray), 3 (black), 4 (green), and 5 (purple). (B) G12D K-Ras with metastable states similarly colored as I (blue), II (gray), III (black), IV (green), and V (purple). The free energy landscape as a function of the two slowest ICs for (C) pTyr32-G12D and (D) G12D K-Ras.

Figure 12.

Network diagram of the five metastable states identified by the Markov state model. The metastable states are represented by circles and arrows indicate the transition probabilities between two states. The structures depict the five metastable states found through the MSM analysis, each circle illustrating 10 representative protein conformations (generated using MSM), which identify also the SI (red) and SII (blue) regions. Key (A) pTyr32-G12D K-Ras; (B) G12D K-Ras. The circle colors are the same as in Figure 11.

Table 1.

Stationary Probability and the Free Energy of Metastable States of G12D K-Ras and Its Phosphorylated Counterpart

pTyr32-G12D K-Ras		
metastable state S_i	π_{S_i} (%)	free energy ($k_B T$)
1	10	2.304
2	3	3.635
3	14	1.969
4	11	2.230
5	63	0.467
G12D K-Ras		
metastable state S_i	π_{S_i} (%)	free energy ($k_B T$)
I	21	1.556
II	39	0.939
III	20	1.610
IV	1	4.282
V	18	1.692

Table 2.

Maximum Four Fluxes Path of G12D K-Ras and Its Phosphorylated Counterpart

pTyr32-G12D K-Ras	
path	percentage (%)
1 → 2 → 4 → 5	42
1 → 4 → 5	24
1 → 2 → 3 → 4 → 5	23
1 → 3 → 4 → 5	12
G12D K-Ras	
path	percentage (%)
I → II → III → V	55
I → II → V	16
I → II → IV → V	14
I → V	8