Association Rate Constants of Ras-Effector Interactions Are Evolutionarily Conserved

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Abstract

Evolutionary conservation of protein interaction properties has been shown to be a valuable indication for functional importance. Here we use homology interface modeling of 10 Ras-effector complexes by selecting ortholog proteins from 12 organisms representing the major eukaryotic branches, except plants. We find that with increasing divergence time the sequence similarity decreases with respect to the human protein, but the affinities and association rate constants are conserved as predicted by the protein design algorithm, FoldX. In parallel we have done computer simulations on a minimal network based on Ras-effector interactions, and our results indicate that in the absence of negative feedback, changes in kinetics that result in similar binding constants have strong consequences on network behavior. This, together with the previous results, suggests an important biological role, not only for equilibrium binding constants but also for kinetics in signaling processes involving Ras-effector interactions. Our findings are important to take into consideration in system biology approaches and simulations of biological networks.

Introduction

Protein-protein interactions are the central elements in all signal transduction processes. The life times of protein complexes as well as regulatory processes need to be tightly controlled for proper systems functioning. Affinities are used to characterize the strength of protein interactions and the affinities between proteins involved in signaling processes have been shown to correlate with the activities (output/response) in such signal transduction processes [1,2]. In the majority of the cases, affinities between proteins and protein-ligands are determined using equilibrium binding methods, like isothermal titration calorimetry and fluorescence based methods, while rate constants of association and dissociation are only rarely determined. However, correlations of either association or dissociation rate constants with in vivo activity suggest that kinetic properties play a role in the cellular context [3–7]. As the affinity (Kd) can be described as the ratio between the dissociation (kₐₐₜ) and association (kₐₜ) rate constants, different ratios of kₐₜ and kₐₐₜ values can give rise to similar affinities. Kinetic rate constants have been shown to be important for signal transduction, however to which extent kinetics influence signaling might depend on the actual network and network topology. We could speculate that fast kₐₜ and kₐₐₜ values could result in rapid activation and deactivation upon short pulses of a stimulus, while slow ones could filter noise and result in prolonged signaling. If this is true it might open new aspects of cellular signal transduction regulation and could probably lead to conceptually new strategies in drug design. It is likely that the answer will depend on the network topology: rate constants might be important in some signaling branches, in others not.

Evolutionary conservation of protein composition and biochemical properties is usually a valuable indication for the cellular importance of a specific protein complex. In this study we have selected the Ras-effector complex formation, in order to analyze whether kinetic rate constants are evolutionary conserved. Ras proteins belong to the Ras superfamily of small GTPases and they have key roles in various signal transduction pathways, like proliferation and differentiation [8]. They act as molecular switches by cycling between an active GTP-bound and an inactive GDP-bound state [9,10]. Active Ras (Ras-GTP) can interact with effector molecules such as the Ser/Thr kinase Raf. The resulting Raf activation triggers the MAP kinase pathway, which leads to the transcription of target genes in the nucleus [11,12]. Other Ras-GTP binding effector proteins that have been identified are the PI3-kinase, members of the RafGDS family, and AF6 [13–16]. Effector proteins bind to Ras-GTP via a common domain with a ubiquitin-like topology [17–22], and various structures of effector domains in complex with Ras proteins have revealed a similar binding mode that involves mainly two antiparallel β-sheets of the RBD and Ras, respectively [23–29].

As Ras-effector protein interactions play a key role in cells, pathways involving Ras-effector interactions can be assumed to be at least partially conserved during evolution. In this study we analyzed whether the affinities and the association rate constants are conserved for 10 Ras-effector complexes in 12 different species, including worms, flies, fishes, and mammalian organisms. We used homology interface modeling and energy calculations, using FoldX 2.8 [http://foldx.crg.es/] [30,31] in order to model Ras-effector interactions of proteins from different organisms. FoldX uses an algorithm based on the original work of Schreiber
Author Summary

Cellular signal transductions processes are based on protein interactions. Proteins can either associate transiently with each other or form stable complexes, and the strength of the interaction is described by the affinity (the affinity is the ratio between the rate of dissociation and association). Protein complexes with similar affinities can bind and dissociate with different rates, and these rates describe the kinetic properties of protein binding. These kinetic rates are important for signaling; however, to what extent individual changes in such rate constants are biologically important or whether the affinity is more crucial might be different in different signaling processes. In this study we analyze whether association rates are conserved during evolution, because evolutionary conservation of protein biochemical properties is usually a valuable indication of its importance. We analyzed the binding of Ras proteins to effector domains, which are central proteins in many signal transduction pathways, in different organisms. On the basis of homology modeling and energy calculations we find that association rates are conserved, although the sequence similarity decreases compared to the human protein. Our finding should encourage further analysis of the importance of kinetics for cellular signal transduction.

Results

Importance of Electrostatic Charge Complementarity for Ras-Effector Association Kinetics

Binding of effector proteins to Ras proteins is mediated via a domain with an ubiquitin-like topology [35]. Members of the ubiquitin domain superfamily are the RA, the RBD, the PISKrbd, the UBQ and the B41/ERM domain families [36]. However, the binding of Ras to effector domains does not depend on the fold itself, but rather on certain amino acid residues on the surface that are crucial for binding.

An important observation found in Ras-effector complex structures is the high charge complementarity between the proteins of the complex, where Ras is mainly negatively charged and the effector RBDs are mainly positively charged [23–29]. Various studies have shown that a strong electrostatic surface complementarity in a protein complex enhances the association rate constant by forming of a low affinity encounter complex before the final high affinity complex is formed [37–42]. The complex formation itself is promoted by electrostatic steering which stabilizes the transition state by decreasing the energy barrier for association [42,43]. In agreement with this concept of electrostatic steering and encounter complex formation, the association rate constants between Ras and effector domains does not depend on the fold itself, but rather on certain amino acid residues on the surface that are crucial for binding.

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FoldX Electrostatic Interactions and Association Rate Constants Correlate with Experimental Association Rate Constants in Ras/Raf and Ras/RalGDS Complexes

Although the algorithm developed by Schreiber and co-workers implemented in FoldX (http://foldx.crg.es/) [30,31], has been validated experimentally on many different proteins, still it is a prediction method and as such needs some validation on the particular system under study. For this, we have selected the Ras-Raf complex and calculated $k_{on}$ values ($\Delta G$) at different salt concentrations, ranging from 0 to 800 mM NaCl (corresponding to an ionic strength of ~50 to 850 mM in 50 mM Tris-buffer), and compared these results with experimental $k_{on}$ values measured at different ionic strength using stopped-flow (Table S1; [48]). The experimental $k_{on}$ values range from 7.4 to 60 $\mu M^{-1} s^{-1}$ and an excellent correlation with calculated association rate constants was observed ($R = 0.99$) (Figure 2A). Further, we used FoldX in order to generate in silico a series of mutations of charged residue in RalGDS, located either in the binding site, or at the edge of the binding site, and we calculated binding energies as well as association rate constants using the Ras-Ral complex. When comparing these results with experiments [43], we find again a very good correlation between experimental and calculated $k_{on}$ values (Figure 2B) ($R = 0.89$, with the slopes of the two correlations [ionic strength and mutants] been similar. This indicates that absolute values of association rate constants can be reliably calculated over a wide range for different ionic strengths and mutations of Ras-effector complexes.
Ortholog Prediction of Ras and Selected Effector Proteins

We have selected proteins containing RA, RBD, PI3Krbd, and B41 domains, similar as in our previous genome-wide Ras-effector homology interface modeling study [34] (Figure 3A), for which binding to Ras has been shown experimentally (Table 1). These include the different isoforms of the Raf kinases, RafGDS, and the related proteins, Rgl1, and Rgl2. Other Ras binding domains are the PI3K-p110 gamma subunit, and Krit. In the following we will often refer to members of the ubiquitin superfamily as UBDs, without differentiating between RA, RBD, PI3Krbd, or B41.

In order to derive Ras and effector protein “interactions” from organisms representing the major eukaryotic branches, we have selected the following species (Figure 3B): Homo sapiens (hs) and Mus musculus (mm) were chosen for mammals, Gallus gallus (gg) for birds, Xenopus tropicalis (xt) for amphibians, Fugu rubripes (fr), and Dario rerio (dt) for fishes, Drosophila melanogaster (dm), Drosophila pseudoobscura (dp), Anopheles gambiae (ag) and Apis mellifera (am) for arthropods, and Caenorhabditis elegans (ce) and Caenorhabditis briggsae (cb) for nematodes. The orthologs were predicted by using the ENSEMBL (http://www.ensembl.org) [49] and the IMPARANOID databases (http://imparanoid.cgb.ki.se/) [50,51]. Domains were predicted using SMART [52,53] and the sequences were aligned automatically and by manual curation taking structural information into account [35] (Figure S1). Depending on the organism, between 22% and 78% of all human proteins orthologs were identified. When taking into account that certain proteins in lower organisms are orthologs of more than one human protein, e.g., RafGDS of C. elegans is also an ortholog of Rgl1 and Rgl2, the number of orthologs in different organisms ranges from 33% to 100%. The alignments of the UBDs of Ras effector proteins show a high similarity within orthologs and often also between different proteins of the same domain family. Furthermore, the similarity within the secondary structures of the RBD is higher than within the loops, indicating a conservation of the binding mode. The sequence identity of ortholog proteins (for detailed description see method) ranges between 100 and ~20% (Table S2). However, in the majority of the cases the sequence identity decreases to ~30/40%. The only exceptions are the different PI3 kinase p110 isoforms, where a drastic drop in sequence identity is observed for the corresponding orthologs/isoforms in C. elegans/C. briggsae.

The sequences of proteins that have a key role in cells are usually highly conserved among all organisms. In accordance with this, the sequences of Ras proteins were found to be nearly identical, especially in the effector binding region (Figure S2). The three Ras proteins, H-Ras, N-Ras and K-Ras could only be found in vertebrates, for arthropods and nematodes there is only one Ras protein which is most likely to be an ortholog of H-Ras. Due to the similarity in the effector binding region, only HRas was modeled (here termed as Ras).

Homology Interface Modeling of Ortholog Ras-Effector Complexes

The first three secondary structure elements (ß1, ß2, and ß3) of the ubiquitin-like domain determine the interaction surface towards Ras and they have the largest impact on binding energy of the complex [33]. In those cases in which a crystal structure of Ras in complex with a RBD domain was available we use the structure to model the ortholog sequences (Ras-Raf, Ras-Ral, Ras-PI3 Kinase, Ras-Byr). For the rest we used the templates modeled in our previous study [34] (Table S3) that were validated experimentally by pull-down experiments (for details see methods). Only those UB domains that could be reliably modeled were selected (e.g., no van der waals’ clashes above a fixed threshold of 2 kcal/mol). The species were then grouped into human (hs), mouse (mm), birds (gg), amphibians (xt), insects (dm, dp, ag, am) and nematodes (ce, cb). The mean of ΔG and ΔG_kin within each group was calculated and taken as value for the complete group. By grouping the different organisms, the problem of missing sequences can be solved for many proteins and mean values as well as standard deviation of ΔG and ΔG_kin can be calculated (the results do not change if we consider individual organisms, data not shown).
The results for all interaction energies ($\Delta$G_{int} FoldX) and contribution of association rate constants ($\Delta$G_{kon} FoldX) were plotted against the divergence time (Table S4 and Figure S3). While the sequence similarity decreases with increasing divergence time, the interaction energies as well as the association rate constants are conserved. A selection of representative results is shown in Figure 4. A comparison of the mean values for all interaction energies and $k_{on}$ values calculated for a particular Ras-effector complex in different organisms shows that the standard deviations are in the majority of the complexes small (Table S5 and Figure 5A). Interestingly, the interaction energies correlate with the association rate constant contribution (Figure 5B).
This indicates that also for the so far kinetically uncharacterized UBD domains, the changes in $\Delta G$ are mainly a consequence of changing $k_{on}$. Thus, this could be underlying binding principle for the complete Ras-effector family.

In order to demonstrate that large changes in association rate constants would have been possible theoretically, we have selected the human Ras-RalGDS complex as an example for an in silico mutagenesis using FoldX. By either introducing positively or negatively charged residues at all positions at the surface of RalGDS, the FoldX-$k_{on}$ contribution could be increased from $-3.65$ kcal/mol to $-7.6$ kcal/mol or decreased to $-0.47$ kcal/mol, respectively (data not shown).

### Simulation of a Minimal Ras-Effector Network

In order to analyze whether compensating changes in $k_{on}$ and $k_{off}$ can influence signal transduction, we used in silico simulations of a sub-network within the EGF signal transduction pathway. Activation of proteins following EGF stimulation is one of the most studied signaling systems, which involves the Ras-CRaf interaction as central elements, and numerous simulation models exist, which are able to correctly predict different aspects of EGF signaling found experi-

### Table 1. Selected Ras binding domains, structural information, and template structures used for modeling.

| Effector RBD | PDB Single Domain | PDB Complex with Ras Protein | Template Structure Used for Modeling |
|--------------|-------------------|-----------------------------|-------------------------------------|
| AF6 RA1      | 1wxa              | 1lfd                        | 1lfd                                |
| AF6 RA2      | 1wxa              | 1lfd                        | 1lfd                                |
| Araf         | 1wxm              | 1gua                        | 1gua                                |
| Craf         | 1rfa, 1rb, 1gua   | 1gua                        | 1gua                                |
| Braf         | 1gua              | 1lfd                        | 1lfd                                |
| Krit1        | 1lfd              |                             |                                     |
| PI3K p110 gamma | 1he8   | 1he8                        |                                     |
| RalGDS       | 1ld, 2rgf         | 1lfd                        | 1lfd                                |
| Rgl1         | 1ef5              | 1lfd                        | 1lfd                                |
| Rgl2         | 1lfd              |                             |                                     |

Figure 4. Homology interface modeling of ortholog Ras-effector complexes. Representative Results for predicted association rate constants. Association rate constants are conserved for Ras-effector complexes in different organisms with increasing divergence time, while the sequence identity is decreasing. Color code: AF6_RA2 (orange), RalGDS (dark blue), BRaf (light blue), CRaf (dark green), and AF6_RA1 (light green).
mentally [54–59]. Based on these earlier models we have constructed a minimal network involving Ras and Raf kinase (Figure 6A and Table S6). This minimal model involves activation of GEF upon stimulation (A), which results in activation of Ras (RasT = RasGTP). Subsequent binding of Raf to RasT activates Raf (Raf_act), which in turn leads to activation of a downstream target (X). Negative regulation was introduced by the GAP catalyzed hydrolysis reaction of RasGTP to RasGDP (RasD). We simulated this network by first applying a constant stimulus of “A” for 500 seconds using the wild type kon and koff values for the Ras-Raf interaction. Then we simulated the network with either 10-fold higher kon and koff rate constants, or 10 fold lower kon and koff (Figure 6B). Minor changes are observed when following X over time (activation peak) for the simulation with 10-fold higher kon and koff compared to the wild type situation. Only the simulation of 10-fold lower kon and koff resulted in a slightly smaller activation peak. However, when simulating the network by applying a pulse of stimulation, of 10 s of “A” (and then removing the stimulus), large changes in the activation peak are observed, with a higher maximum for the simulation of 10-fold higher kon and koff values for the Ras-Raf interaction (Figure 6C). This shows, that under certain cellular conditions, like short pulse of activation, large changes in activation are expected for mutants with similar affinity, but changed and compensating effects on kon and koff. Thus, kinetic properties can be crucial, and in the case of Ras-effector interactions, association kinetics will be important to result in sufficient activation, when the system is activated by applying a pulse.

Discussion
The complex formation of Ras and effector proteins is driven by high association rate constants and only moderate dissociation rate constants [45–47]. Further, changes in affinity are mainly the consequence of changed association rate constants. Association

Figure 5. Mean values and standard deviation for calculated interaction energies (ΔGint) and association rate constants (ΔGkon).

(A) Results from UBDs from the same protein or from the same protein family were shown in similar color. The results were taken from Table S5. (B) Correlation of calculated interaction energies with calculated association rate constants. The results were taken from Table S5, and the correlation coefficient is 0.71. doi:10.1371/journal.pcbi.1000245.g005
rate constants can be influenced by mutating charged residues at the edge of the interface [32,43]. If electrostatic interactions and association rate constants are important for the biological function of the cell, they should be conserved during the course of evolution. Using homology modeling and energy calculation covering a wide-range of sequences, and relating the output to the sequence conservation, we found that interaction energies as well as the electrostatic contributions and the association rate constants are conserved as well. While the sequence identity decreases with divergence time between the selected organisms, no trend could be found for the interaction energy and energies related to the electrostatics and kon, although theoretically it should be possible, when sampling the possible contributions of kon at different amino acid positions (Figure S4).

Biologically, electrostatic interactions within Ras-effector complex interfaces could be functionally important, because they are the basis for the observed dynamic behavior, as observed in the case of Ras binding to the Raf kinase effector protein: The Ras-RafRBD complex formation is characterized by both high association and dissociation rate constants (kon and koff), leading to affinities (Kd = koff/kon) in the range of 1 to 0.05 μM, under physiological conditions (this relatively low affinity seems to be functionally sufficient, since Ras is attached to the membrane via a lipid modification). The high kon values provide the possibility to have a fast dissociation of the complex, while still having a reasonable tight binding complex (the lifetime of the complex between Ras and RafGDS, for example, is 0.1 s-1; see reference [47]). As Ras signaling depends very crucially on a strict control through regulating proteins like GAPs (GTPase activating proteins) and GEFs (guanine nucleotide exchange factors), this fast dissociation allows regulatory proteins to access and act.

We assume that electrostatics contributions and binding kinetics could be important in other Ras signaling pathways, since association rate constants were found to be conserved during evolution, as demonstrated in this study for 10 effector domains. Further in vivo analysis will be needed to prove this hypothesis. These experiments could be performed by designing mutant variants, which are expected to have similar affinities, but changed association and dissociation rate constants. These protein variants could be expressed in cells and the effect on signal transduction monitored, e.g., after different pulses of stimulation.

It is expected that the effect of changing rate constants depends also on the network topology (negative feedback, feed forward inhibition, etc). This knowledge will be important for systems biology and simulation approaches, in order to know, at which positions in the network affinities will be sufficient, while for other accurate rate constants will be crucial for correct prediction. Further, it could open conceptually new aspects in drug design.

Methods

Selected Species, Orthologs, and Domain Prediction

Proteins from the following species were used in order to get a good representation of all branches: Homo sapiens and Mus musculus (mammals), Gallus gallus (birds), Xenopus tropicalis (amphibians), Fugu rubripes and Danio rerio (fishes), Drosophila melanogaster, Drosophila pseudoobscura, Anopheles gambiae and Apis mellifera (arthropods), and Caenorhabditis elegans and Caenorhabditis briggsae (nematodes). Only
two RA domain containing were retrieved from *Saccharomyces cerevisiae* (sc), because these proteins are involved in a different pathway. For each protein, the human ENSEMBL protein ID was retrieved from ENSEMBL [49]. The orthologies were predicted by using the ENSEMBL database for *Xenopus tropicalis*, or the INPARANOID database [http://www.inparanoid.cgb.ki.se] [50,51]. ENSEMBL [49] classifies the prediction based on the BLAST results. Only those orthologs were chosen that were a unique best reciprocal hit in both directions. As the INPARANOID database [50,51] provides more information about orthologs of members of protein families, e.g., PI3K p110, the prediction was preferentially used. The sequences retrieved from ENSEMBL or INPARANOID were analyzed using SMART [http://smart.embl-heidelberg.de] [52,53], in order to determine the domain architecture of the protein and the domain sequences.

Homology Modelling and Energy Calculations Using FoldX

For modeling of Ras-binding domains in complex with Ras proteins, we have taken the pdb-files of the following Ras effector complexes: Ras-RalGDS (pdb-entry: 1LFD), Ras-PI3KInaase (pdb-entry: 1HE8), and Raps-Raf (pdb-entry: 1GUA). Different template structures have been generated by deleting certain parts in the complex; the decision was mainly based on the alignment used to model the different binding domains. The orthology sequences for one protein were aligned using standard automatic alignment tools, since sequence homology is high. However, the alignment of different effector domains from different families (RA, RBD, PI3Krbd, B41), was done based on manual curated structural-based sequence alignments as discussed in detail in a previous publication [35]. Basically two kinds of template structures have been generated (Table S3): a short version, where all secondary structure elements and loops (apart from B1, B1, B2, a1) were deleted, as this is the part mainly contributing to the binding energy (similar as done in our previous study [33,34]). In addition ‘long template’ structures have been generated. We could not model loop regions in those cases where the loops where not of the same length. For having a proline at the beginning of β-strand 1 (position 26 in RalGDS, position 229 in PI3K, position 66 in Raf and position 81 in spByr2), we prepared special template structures by moving the backbone slightly, after introducing the proline at these positions (we checked that the proline was in acceptable dihedral angles and that the main chain CO group was still H-bonded to Ras). These template structures were then used to model the complex structures for AF6_RA2. The homology modelling was done as described before [33,34]. The homology modeling was done based on the sequence alignment (Figure S1 and Figure S2), using different template structures using the design option in a new version of FoldX [30,31]. During this design procedure, FoldX is testing different rotamers and allows neighbor side chains to move. After reconstruction, all models have been passed through an additional optimization step by using the repair function of FoldX [detailed description in [33,34]]. Energy calculations of Ras-effector complexes have been done using FoldX as described before [http://fold-x.crg.es] [30,31].

Simulations of a Minimal Ras-Effector Network

A model was generated based on previous models of EGF signal transduction (see Table S6). Simulations were performed using the SmartCell software [http://smartcell-crg.es] [60] using ordinary differential equations.

Supporting Information

**Figure S1** Alignment of UB domains

Found at: doi:10.1371/journal.pcbi.1000245.s001 (0.05 MB PDF)

**Figure S2** Alignment of Ras proteins

Found at: doi:10.1371/journal.pcbi.1000245.s002 (0.03 MB PDF)

**Figure S3** Diagrams of DGint and DGkon FoldX values plotted against the divergence time

Found at: doi:10.1371/journal.pcbi.1000245.s003 (0.07 MB PDF)

**Figure S4** FoldX mutational scanning of RafRBD and RalGDS-RA. (A) Ras-RaRBD (pdb-entry 1GUA). (B) Ras-RalGDS (pdb entry 1LFD). Effect of all residues in Raf-RBD or RalGDS-RA on the contribution of DG kon as calculated by FoldX. Either positively charged residues were mutated to alanine (red) or negatively charged or neutral residues were mutated to lysine using FoldX and the difference compared to the WT DGkon was calculated and plotted for every amino acid position. Found at: doi:10.1371/journal.pcbi.1000245.s004 (0.03 MB PDF)

**Table S1** Experimental and calculated association rate constants

Found at: doi:10.1371/journal.pcbi.1000245.s005 (0.03 MB PDF)

**Table S2** Divergence times and sequence identities for Ub domains

Found at: doi:10.1371/journal.pcbi.1000245.s006 (0.02 MB PDF)

**Table S3** Template structures used for homology modelling

Found at: doi:10.1371/journal.pcbi.1000245.s007 (0.01 MB PDF)

**Table S4** FoldX results for all homology models

Found at: doi:10.1371/journal.pcbi.1000245.s008 (0.01 MB PDF)

**Table S5** Mean values and STDEV for homology models

Found at: doi:10.1371/journal.pcbi.1000245.s009 (0.01 MB PDF)

**Table S6** Modelling parameters

Found at: doi:10.1371/journal.pcbi.1000245.s010 (0.05 MB PDF)

Author Contributions

Conceived and designed the experiments: LS. Performed the experiments: CK DA. Analyzed the data: CK DA LS. Wrote the paper: CK LS.
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