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Biacore™ systems for label-free interaction analysis

Deeper insights into biological realities

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Detection of interactions

Interactions between biomolecules are the key drivers of biological processes. For example, protein-protein interactions ensure signal transduction across membranes via G protein-coupled receptor (GPCR), maintain the structure of complexes by chaperones, or enable the enzymatic modification of proteins via post-translational phosphorylation. Hundreds of thousands of interactions have been identified and collected in databases, for example IntAct (http://www.ebi.ac.uk/intact/). Such a database allows the interactions to be assembled into pathways and studied further. Yet the majority of protein interactions probably remain undiscovered.

Homing in on the nature of interactions

GPCRs are transmembrane receptors that sense molecules outside the cell and activate signal transduction pathways, leading to cellular responses. The work of Suzuki and co-workers (1), based at Tokyo University, has increased the understanding of the interactions and guanine-nucleotide-dependent conformational changes involved in transient GPCR-signaling. Their work represents a typical approach to the study of interactions, in this case between LARG, a guanine nucleotide exchange factor for Rho, and $G\alpha_{13}$, a GPCR subunit. The study starts with detailed interaction analysis, and concludes by looking at the thermodynamics of conformational changes.

Which domains interact?

The first step for Suzuki and co-workers was to determine if interactions occur, and their dependency on effector molecules. They used a cell-based assay with cells transfected with *myc*-tagged deletion constructs of LARG with different combinations of domains, and $G\alpha_{13}$. They showed that $G\alpha_{13}$ interacts directly with LARG through its RH domain, DH/PH domains, and C-terminal region (Fig 1). The next step was to characterize these interactions with SPR-based assays using Biacore 3000.

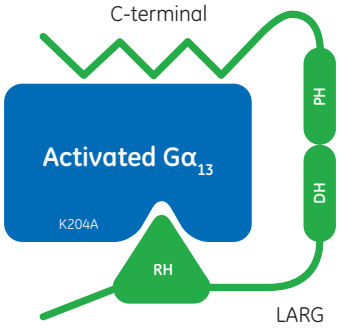

Fig 1. Leukemia-associated RhoGEF (LARG) is a guanine nucleotide exchange factor for Rho and has a number of domains that can interact with $G\alpha_{13}$:

RH: Regulator of G protein Signaling (RGS) domain, which is required for activity.

DH: Dbl homology (DH) or RhoGEF domain. This consists of an approximately 150 amino acid region that induces LARG to displace GDP.

PH: Pleckstrin homology domain. This domain can bind phosphatidylinositol of membranes and thereby recruit proteins to be directed to cellular compartments or interact in signal transduction pathways. The PH domain can increase catalytic efficiency.

The position of the K204A mutation in the RH-binding region of $G\alpha_{i/13}KA$ is also shown.

SPR-based analysis confirms the interactions and provides more detail

Studies were based on two variants of $G\alpha_{13}$: $G\alpha_{i/13}$, a chimera of $G\alpha_{13}$, and $G\alpha_{i/13}KA$, which has a mutation, K204A, in the domain that recognizes the RH domain of LARG. These variants could be activated with AMF ($AlCl_3$, $MgCl_2$, and NaF).

The variants of $G\alpha_{13}$ were immobilized on the surface of Sensor Chip CM5 using amine coupling chemistry, and activated by including AMF in the running buffer (Fig 2). Solutions of LARG constructs at different concentrations were flowed over the sensor surface in the presence or absence of AMF, followed by a regeneration buffer.

The results for $G\alpha_{i/13}$ clearly showed that AMF activation is necessary for all interactions and that these involve RH, DH, and PH domains of LARG. Comparing LARG construct DPC (DH, PH, and C-terminal) and DH/PH (DH and PH domains, only) indicated that the C-terminal is also involved. Suzuki and co-workers also used $G\alpha_{i/13}KA$ to confirm that the mutation at Lys-204 (K204A) significantly reduced the affinity of $G\alpha_{13}$ for the RH domain of LARG, without affecting interactions with the DH and PH domains.

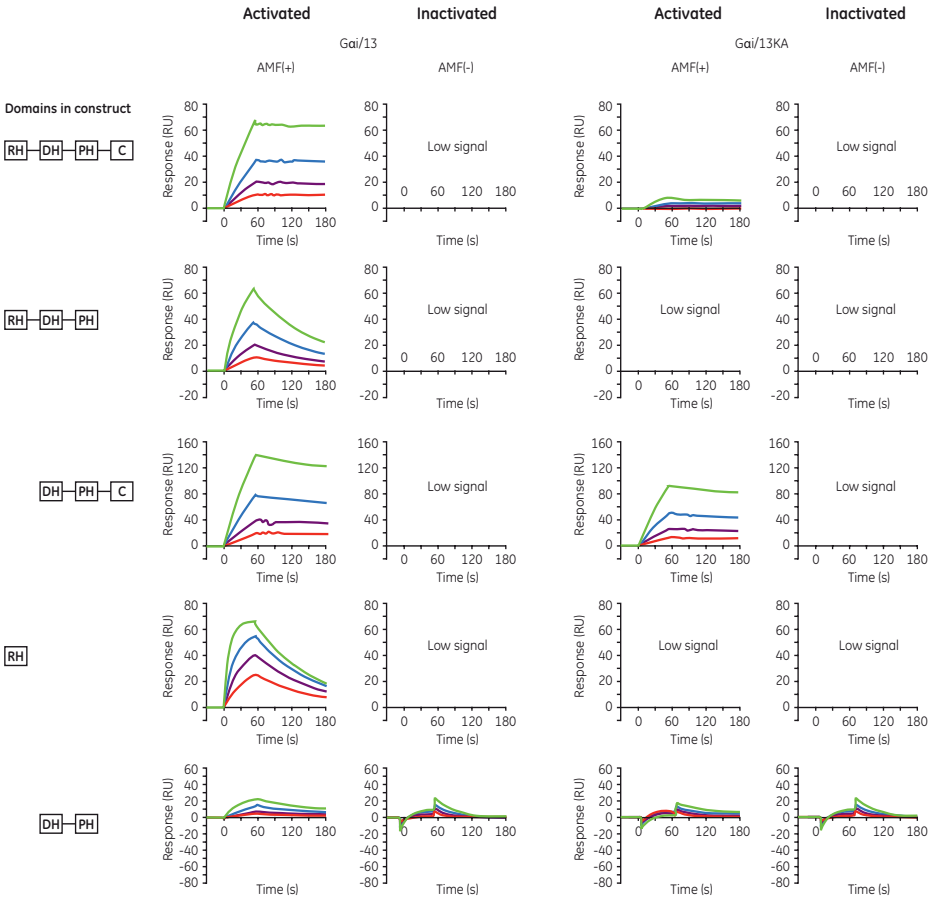

Fig 2. Kinetics of binding of LARG to $G\alpha_{i/13}$ or $G\alpha_{i/13}$KA immobilized on a sensor surface and analyzed using Biacore 3000. $G\alpha_{i/13}$ and $G\alpha_{i/13}$KA proteins were immobilized on Sensor Chip CM5 on separate spots. The concentrations of proteins were in the nM to µM range, depending on the protein. (Adapted from Figure 1C in Suzuki, N. *et al., J. Biol. Chem.* **284**, 5000–5009 [2009]).

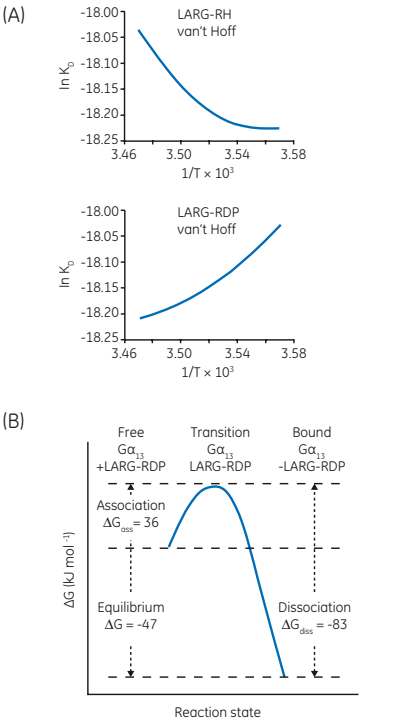

Fig 3. Thermodynamic analysis of $G\alpha_{13}$-LARG interaction. (A) Thermodynamic analysis of the $G\alpha_{13}$-LARG complex formation and dissociation through its RH and DH domain. van't Hoff plots of the experimental data are shown. (B) Schematic reaction profile of the thermodynamic energies at the different states of $G\alpha_{13}$-LARG interaction. The thermodynamic parameters at an equilibrium state and at a transition state were estimated from van't Hoff plots and Eyring plots. (Adapted from Figure 4 in Suzuki, N. *et al., J. Biol. Chem.* **284**, 5000–5009 [2009]).

Determining the extent of the interaction

The raw data represented in these sensorgrams was used to determine kinetic data: association rate constant (k_a), dissociation rate constant (k_d) and equilibrium constant (K_D). The relative affinity (in parentheses) of the LARG constructs for $G\alpha_{i/13}$ increased in the order RH (×1) < RH-DH-PH (×2) < RH-DH-PH-C terminal (×20). The interaction involving the C-terminal therefore decreased the dissociation rate of the $G\alpha_{i/13}$-LARG complex considerably. The association of the RH domain with the $G\alpha_{13}$ surface, including Lys-204, probably induces the conformational change of the DH/PH domains of LARG necessary for its biological action.

What are the dynamics?

Suzuki and co-workers decided to investigate the dynamics of the conformational changes induced by $G\alpha_{13}$-LARG binding (especially the contribution of the RH-domain and the DH/PH domains) by determining the thermodynamics of these interactions. This involved using Biacore T100 to analyze the interactions between LARG and $G\alpha_{i/13}$ immobilized on Sensor Chip CM5 (Fig 3).

The interaction between RH-DH-PH and $G\alpha_{13}$ had a higher free energy change than the RH/$G\alpha_{13}$ interaction, which is consistent with the higher affinity of the LARG-RH-DH-PH construct compared with the LARG-RH construct (Fig 3). Of particular interest was the fact that the $G\alpha_{13}$-RH interaction is enthalpy-driven and entropically unfavorable. This contrasted with the binding of the RH-DH-PH construct to $G\alpha_{13}$, which is less favorable than LARG-RH with respect to enthalpy, but is entropy-driven with a positive ΔS^0. In general, a large negative heat capacity change (ΔC_p^0) in a protein-protein interaction indicates the removal of water-accessible hydrophobic surface area coupled to conformational changes. Indeed, the researchers were able to conclude that the interaction between Gα13 and the RH domain of LARG triggers conformational changes that bury an exposed hydrophobic surface to create a large complementary surface, thereby promoting complex formation.

Kinetics

The cell is a dynamic system. So, identifying interactions is just the first step in interaction analysis and most often leads to an investigation of kinetics, to answer the following questions:

- How fast do molecules bind (association)?
- How fast do complexes fall apart (dissociation)?

Kinetics therefore determine whether a complex forms or dissociates within a given time span. Association and dissociation measurements can also be used to determine how much complex is formed at equilibrium (the steady state where association balances dissociation). This is the affinity of the interaction.

A stable basis for kinetics studies

Determining the residence time or half-life of a drug on its target receptor is a critical point in drug discovery. SPR-based assays are routinely used in biophysical screening of soluble drug targets to determine equilibrium binding constants, kinetic rate constants and thermodynamic parameters. It becomes more challenging to analyze membrane protein targets, which can be destabilized by detergent-extraction and lose their native ligand-binding capability. This problem is particularly acute in the study of GPCRs, a very important group of drug targets. One method for engineering stability into receptors, called Stabilized Receptor, or StaR™, has enabled the study of receptors that were previously difficult to analyze, including GPCRs. These receptors can be purified in large quantities, retain correct folding, and are stabilized to such an extent that they can readily be used in binding assays. Robertson and co-workers at Heptares Therapeutics in the UK, have used SPR-based assays to analyze the kinetics of interactions between drug candidates and StaR molecules (2).

Designing in protein stability

The researchers first determined the success of mutagenesis by determining the thermostability of over three hundred mutations, and combinations of mutations with a method involving stabilization with a radioactive agonist. Stability with respect to detergents was also tested. The most promising candidates were then selected for recombination and kinetic analysis.

Determining kinetics with more accuracy and throughput

The kinetics of interactions involving GPCRs are usually determined by measuring dissociation rates of a radioactive ligand in the presence or absence of the test compound. Having confirmed that radio-ligand binding studies and SPR-based assays gave similar results for affinity, Robertson and co-workers found that SPR-based assays enabled analysis of kinetics on solid-phase and thereby provided a simpler, higher throughput, and more accurate method compared to conventional methods.

The researchers used SPR to examine the kinetics of one stabilized construct, A_{2A}-Star2, in more detail. The kinetic characterization of five A_{2A} ligands, with relative molecular masses in the range 285 to 345, using SPR detection assays is shown in Figure 4. The binding was concentration dependent, and showed high reproducibility between triplicates. Binding parameters could be determined after fitting the sensorgrams to a 1:1 model. The range of association and dissociation rate constants displayed by these antagonists gave a 10 000-fold range in affinities. In addition, the validity of the approach was confirmed by the fact that affinity constants closely correlated with those obtained in equilibrium binding studies on the wild-type receptor in membranes.

The ability to stabilize receptors opens up new possibilities for the analysis of relatively unstable GPCRs, using crystallography to probe structure, and SPR to analyze kinetics. This approach should lead to the identification of ligand candidates with the desired interaction properties.

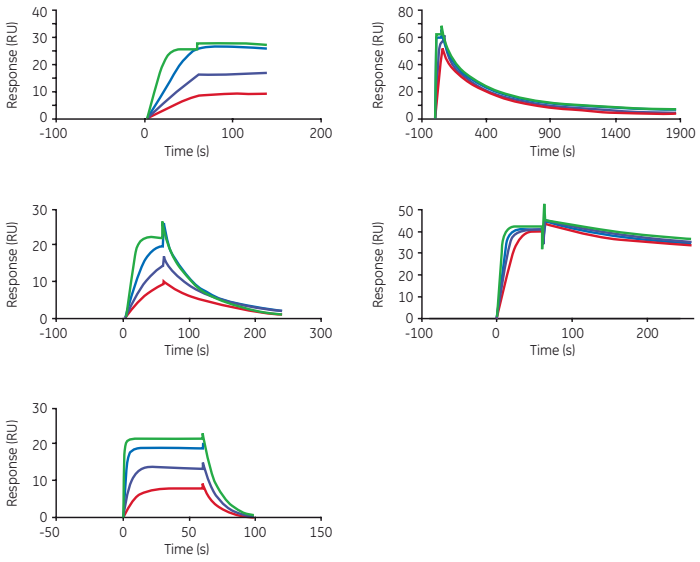

Fig 4. Kinetic characterization of five A_{2A} ligands using SPR. A_{2A} StaR2 was immobilized on Sensor Chip CM5 using amine coupling. On- and off-rates of antagonist binding were then determined for each compound, (Adapted from Figure 6 in Robertson, N. *et al., Neuropharmacology* **60**, 36–44 (2011)).

Structure and function

The structure of a biomolecule is usually critical to its function. The structure may be static, but is more often dynamic and influences, or is influenced by, interactions with other biomolecules. Very often, as in the case of enzymes, changes in structure are instrumental in the function.

Protein structure may be determined by sequencing and then applying biophysical methods such as X-ray crystallography and NMR to determine the three-dimensional, structure in interaction with cofactors where necessary. Understanding the roles played by conformational changes and structure in interactions requires application of other methods, such as biochemical assays and biophysical techniques, such as SPR-based assays.

Find out how proteins work by looking at their domains

The three-dimensional structure of a protein can be determined from its sequence and by applying biophysical techniques, such as X-ray crystallography and NMR spectroscopy. The structural domains within the protein can then be dissected out and isolated, which then makes it possible to apply SPR-based assays to investigate interactions between domains. This deepens the understanding of the dynamics that link structure and function. Luna-Vargas and co-workers, based at The Netherlands Cancer Institute, have applied this approach in the study of an enzyme involved in the processing of ubiquitin (3).

Ubiquitin (Ub) is a small, ubiquitous regulatory protein with a number of functions, including being used by conjugating enzymes to label proteins that are to be processed. This discovery led to the award of the Nobel Prize for chemistry to Aaron Ciechanover, Ayram Hershko, and Irwin Rose in 2004. Deubiquinating enzymes (DUBs), such as USP4, balance this labeling activity by removing Ub from the target molecule. Luna-Vargas and co-workers used SPR detection to investigate the structural elements of ubiquitin-specific protease 4 (USP4) that control its deubiquinating activity.

Determining the structural elements to be isolated

The researchers first determined the structure of the catalytic domain of USP4, which consists of two regions, D1 and D2, using X-ray crystallography. They also prepared a number of constructs comprising various combinations of the protein domains - for example a construct with an insert between D1 and D2 that contained an Ubiquitin-like domain (Ubl), as in the native protein. In vitro de-ubiquitinating assays indicated that including the Ubl-insert in USP4-D1D2 (USP4-D1-Ubl-insert-D2) reduced catalytic activity, which suggested that the Ubl-insert inhibits the DUB activity of USP4 through an autoregulatory mechanism.

Exploring the interactions

The researchers then used SPR-based assays to determine if the Ubl-insert interacts directly with the catalytic domain, USP4-D1D2. GST (glutathione S-transferase)-fused fragments were immobilized on α-GST antibodies that had been lysine-coupled to Sensor Chip CM5 (Fig 5A). USP4D1-D2 was flowed over the surface and interactions were detected using Biacore T100. Luna-Vargas and co-workers showed that the affinities of the Ubl-insert, and Ubl alone, for USP4-D1D2 are indeed similar to that of Ub itself (Fig 5B-D).

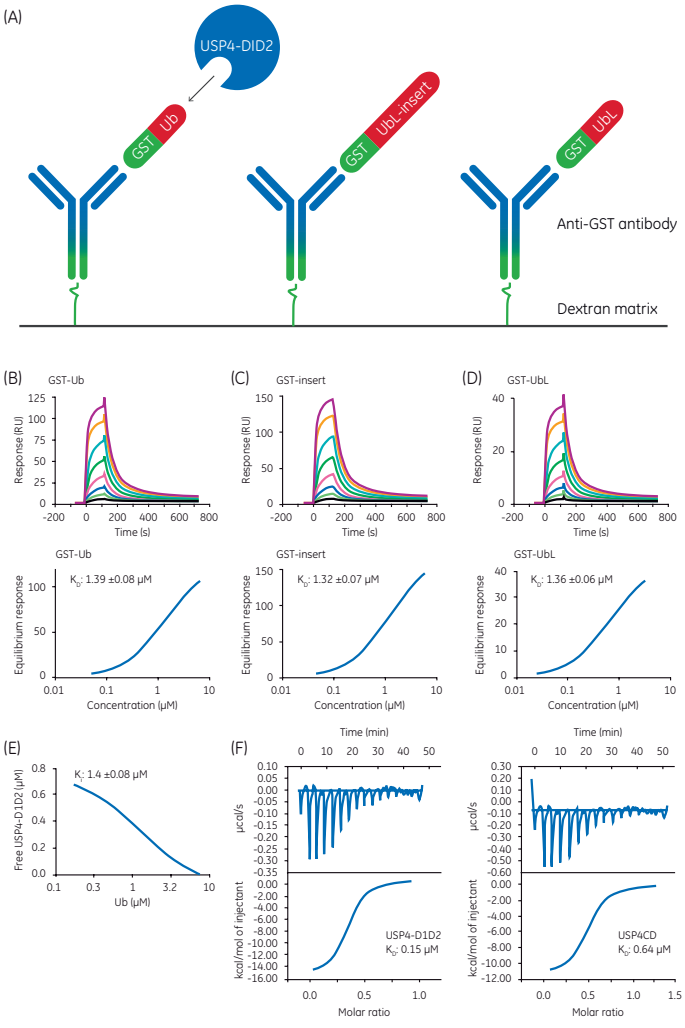

Fig 5. Ubiquitin competes with the insert or Ubl-domain for binding to USP4–D1D2. (A) GST-fused fragments were immobilized on α-GST antibodies that had been immobilized on a sensor chip and USP4D1-D2 was then flowed over the sensor surface. (B-D) Interaction of Ub and the insert fragments with USP4–D1D2 was analyzed by SPR-based assays. Top: (B) GST-tagged Ub, (C) GST-insert and (D) GST-Ubl domain. Bottom: Langmuir binding curves. (E) Competition experiment with immobilized GST insert on USP4–D1D2 with varying concentrations of Ub. A one-site competition-binding model was fitted (Ki 1.4 μM). (F) The interaction of Ub with USP4–D1D2 (left) and with full-length USP4CD (right) was studied by ITC. Thermodynamic values were: USP4–D1D2 (ΔH = -14.3 kcal/mol and ΔS = -16.9 cal/mol/deg), for USP4CD (ΔH = -11.4 kcal/mol and ΔS = -10.0 cal/mol/deg) (Adapted from Figure 3 in Luna-Vargas, M .P. *et al., EMBO Rep.* **12**, 365–72 [2011]).

Ubl competes with Ub

SPR-based detection was used in a competition assay, involving flowing USP4-D1D2 over the immobilized Ubl-insert in the presence of increasing amounts of Ub. The data could be fitted to a one-site competition binding model, showing that the USP4 insert containing Ubl competes with Ub for binding to the catalytic domain, USP4-D1D2. Additional experiments, using Isothermal Titration Calorimeter, showed that the Kd values of the constructs D1-Ubl-insert-D2, and USP4-D1D2 for Ub were comparable.

Data from SPR-based assays indicated that Ub binds to D1-Ubl-insert-D2 with slower off- and on-rates than when binding to USP4-D1D2. The researchers concluded that the insert prevents both rapid binding and rapid release of the Ub substrate, thus allowing competitive binding. The similarity of the KD values for purified Ubl and Ubl-insert (Fig 5B-D) suggested that the Ubl domain is the functional part of the insert. SPR-based analysis was also used to demonstrate that the Ubl domain could bind to the catalytic domain of other DUBs. Luna-Vargas and co-workers summarized their data in a model in which DUB activity is partially inhibited by the Ubl domain, which binds to the Ub-binding region of USP4, thus preventing Ub substrate binding (Fig 6).

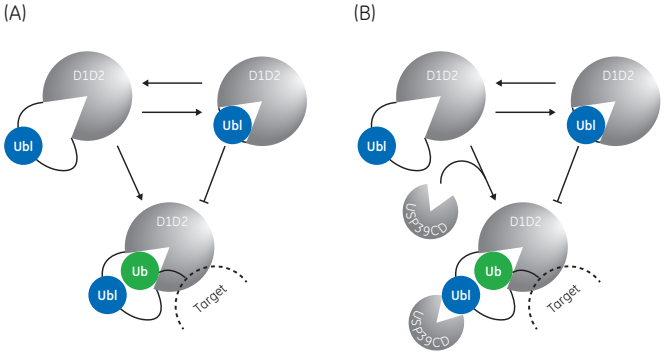

Fig 6. Model for Ubl domain inhibition on USP4. (A) Schematic model of the auto-inhibitory role of the Ubl domain in USP4. (B) Other USP enzymes, such as USP39, may relieve the inhibition by binding to the Ubl domain (Adapted from Figure 5 in Luna-Vargas, M .P. *et al., EMBO Rep.* **12**, 365-72 [2011]).

Explore more

What do you need to get the confidence to take the next step in your research or to submit your next paper for publication? Just imagine what a better understanding of molecular function and activity could do for your research.

Biacore systems can provide key data in real-time to discriminate crucial differences in affinity, even for interactions where challenging targets are involved.

Biacore systems are designed to help you to generate decisive, information-rich data, that will help answer key questions concerning the nature of binding.

References

1. Suzuki, N. *et al.* Activation of Leukemia-associated RhoGEF by G13 with Significant Conformational Rearrangements in the Interface. *J. Biol. Chem.* **284**, 5000–5009 (2009).
2. Robertson, N. *et al.* The properties of thermostabilised G protein-coupled receptors (StaRs) and their use in drug discovery. *Neuropharmacology* **60**, 36–44 (2011).
3. Luna-Vargas, M .P. *et al.* Ubiquitin-specific protease 4 is inhibited by its ubiquitin-like domain. *EMBO Rep.* **12**, 365–72 (2011).

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