High Throughput Assays for the Characterization of Monomeric GTPases of the Ras Superfamily

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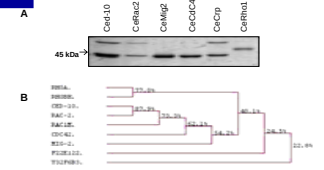
1 Abstract

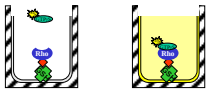
We have developed an innovative analytical platform to perform systematic comparison of protein families based on their biochemical properties. Our approach was validated on the Rho subfamily of GTPases. We used two high throughput technologies (AlphaScreen™ and FlashPlate®), to measure nucleotide binding capacity, exchange, and hydrolysis activities of small monomeric GTPases. These two technologies benefit from high sensitivity while allowing for homogenous and high throughput assays. To analyze and integrate the data obtained, we have developed an algorithm that allows the classification of GTPases according to their enzymatic activities. Integration and hierarchical clustering of these results revealed unexpected features of the small Rho GTPases when compared with primary sequence-based trees. We therefore suggest a novel phylobiochemical classification of the Ras superfamily of GTPases.

2 RhoGTPases from C.elegans



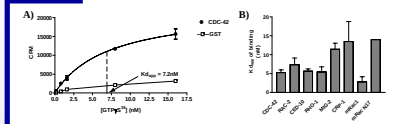
A) SDS-PAGE of RhoGTPases GST-fusion proteins. Cloning was performed using Gateway from Invitrogen. **B)** A Rho GTPases phylogenetic tree was produced by multiple sequence alignments analysis with ClustalW followed by DNAsis. All GTPases except RhoBH and Rac1M are found within the C.elegans genome.

3 GTP loading activity



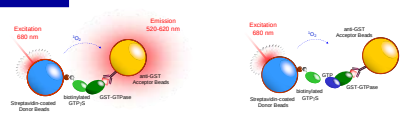
[^{35}S]GTPγS saturation binding assay. FlashPlate® experimental design for [^{35}S]GTPγS binding assay. Nucleotide-free GST-GTPases were added to a GSH FlashPlate and incubated with different concentrations of [^{35}S]GTPγS. Radioligand interacting with the GST-GTPase activates the FlashPlate scintillation and generate a luminescence.

4 GTP loading activity



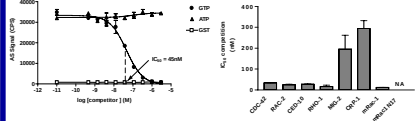
A) Saturation curve obtained with the binding assay shows the kinetics of association between the [^{35}S]GTPγS and GST-CDC-42 or GST alone. The apparent Kd (Kd_{app}) value corresponds to the concentration at which 50% of the maximum binding is reached. This graph represents a single experiment done in triplicate. **B)** comparison of the Kd_{app} values obtained in the [^{35}S]GTPγS loading assay for six *C. elegans* GTPases including a GTP binding-deficient GTPase (mRAC-1N17). All the Kd_{app} values represent the average of at least three independent experiments performed in triplicate.

5 GTP competition activity



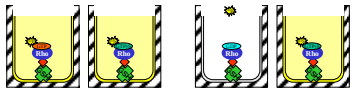
biotin-GTPγS competition assay. AlphaScreen experimental design for the biotin-GTPγS competition assay. Nucleotide-free GST-GTPases were incubated with a constant concentration of biotin-GTPγS and increasing concentrations of unlabeled competitors. Streptavidin Donor beads recognize biotin-GTPγS, and the anti-GST Acceptor beads bind to GST-GTPases. When biotin-GTPγS interacts with the protein, the two beads are in close proximity and a signal is detectable upon laser excitation of the Donor bead. Presence of unlabeled nucleotides (GDP, GTP) competes the binding of the biotin-GTPγS tracer to the GST-GTPase producing a signal decrease.

6 GTP competition activity



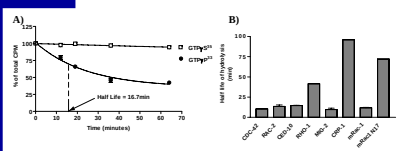
A) biotin-GTPγS competition curves obtained with either ATP or GTP on CDC-42 or GST. The curves are the result of a single experiment done in triplicate. The IC_{50} value corresponds to the competitor concentration required for the loss of 50% of the maximum signal (obtained in the absence of competitor). **B)** comparison of the IC_{50} values obtained for six *C. elegans* GTPases including a GTP binding-deficient GTPase (mRAC-1N17) using the biotin-GTPγS competition assay. The IC_{50} values for the competition assay represent the average of at least three independent experiments performed in triplicate.

7 GTP hydrolysis activity



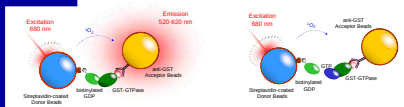
[^{33}P]GTP hydrolysis activity assay. FlashPlate experimental design for the [^{33}P]GTP hydrolysis activity. GST-GTPases were added to a GSH FlashPlate and were loaded with either 500 nCi of [^{35}S]GTPγS or 500 nCi of [^{33}P]GTP. When the GTPase hydrolyzes the γ phosphate of [^{33}P]GTP, the radioactivity is liberated in the reaction mixture, and β particles are quenched by the aqueous solution leading to the inactivation of the scintillation reaction. A decrease of signal is then measured. [^{35}S]GTPγS, a non-hydrolyzable analog of the GTP, is used as a control to measure the nucleotide dissociation from the GST fusion protein.

8 GTP hydrolysis activity



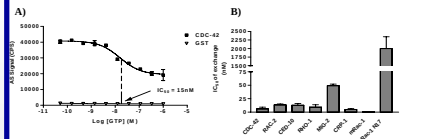
A) the half-life value is the time needed for a GTPase to hydrolyze 50% of the initially bound [^{33}P]GTP with the [^{35}S]GTPγS dissociation parameter excluded. The graph shows the hydrolysis and dissociation curves obtained for CDC-42 in a single experiment done in triplicate. **B)** comparison of the half-life values obtained for six *C. elegans* GST-GTPases including a GTP binding-deficient GTPase (mRAC-1N17) using the hydrolysis activity assay. The half-life values represent the average of at least three independent experiments performed in triplicate.

9 GDP exchange activity



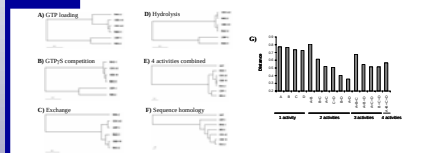
biotin-GDP/GTP exchange activity assay. AlphaScreen experimental design for the measure of the GTPase exchange activity. GST-GTPases were loaded with biotin-GDP and incubated with different concentrations of GTP. Streptavidin Donor beads capture biotin-GDP while the anti-GST Acceptor beads bind to GST-GTPases. When biotin-GDP interacts with the GTPases, the two beads are brought in close proximity and a signal is produced upon laser excitation of the Donor beads. When biotin-GDP is exchanged to GTP, the distance between the beads are no longer in proximity and a loss of signal is measured.

10 GDP exchange activity



A) curves obtained in a single assay done in triplicate for GST-CDC42 and GST alone using this biotin-GDP/GTP exchange assay. The IC_{50} value corresponds to the GTP concentration where 50% of the biotin-GDP is exchanged to a GTP. **B)** comparison of the IC_{50} values obtained for six *C. elegans* GTPases including a GTP binding-deficient GTPase (mRAC-1N17) using the biotin-GDP/GTP exchange assay. The IC_{50} values for the exchange activity correspond to the average of at least three independent experiments performed in triplicate.

11 Rho-GTPase Clustering



Comparison and integration of the enzymatic trees of Rho GTPases. Hierarchical clustering trees based on GTPase biochemical activities are shown (A-D) and according to the integration of these four activities (E). These trees were then compared with the amino acid sequence- based tree (*F*). The bar graph (*G*) shows the distance, calculated using Euclidean matrices, between the SB tree and different hierarchical trees integrating one, two, three, or four activities (*A, B, C,* and *D*). The smaller the distance, the closer the biochemical tree is from the SB tree.

12 Summary & Perspectives

Four (4) different high throughput assays were developed to measure nucleotide binding, exchange and hydrolysis activities of small G proteins of the Ras family.

All the assays developed with both AlphaScreen™ and FlashPlates® were shown to be sensitive, reproducible, robust and suited for the high throughput characterization of small G-proteins.

The comparative analysis of *C.elegans* GTPases revealed that, despite of their sequence homology, all GTPases have distinctive properties based on their biochemical activities.

Results obtained allowed to propose a new classification of small G-proteins based on their enzymatic properties which provides more pertinent information directly linked to protein function.

Using the methods described herein, we are currently characterizing all the 61 C.elegans GTPases at once. All proteins will be compared and classified according to their inherent biochemical activities. A new cluster tree will be built accordingly.