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Reverse-Proteomic Analysis of Rho GTPase Regulation by RhoGAPs using AlphaScreen™

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1 Abstract

The Rho family of GTPases regulates multiple cellular processes including cell cycle progression, cell migration, actin cytoskeleton reorganization, and intracellular membrane trafficking. This broad spectrum of activities implicates Rho GTPases as key components in numerous physiologic and pathologic processes. According to their relative high number of regulators per GTPase, Rho GTPases appeared to be the small G proteins of the Ras superfamily subjected to the strongest time-dependent subcellular distribution. However, the high degree of redundancy of these regulators in higher eukaryotic organisms such as human and mouse limits the feasibility of studying such regulatory mechanisms within these organisms. In contrast, C. *elegans* constitutes an excellent model to study such regulatory mechanisms. Therefore, we propose a systematic and combinatorial analysis of Rho GTPases regulation in order to rule out the complexity of molecular machines regulating Rho GTPases in C. *elegans*.

Complementary DNAs encoding Rho GTPase (7), GAP (21) and GEF (22) domain containing proteins were extracted from the C. *elegans* ORFeome and cloned. At first, GTP and GDP binding to a C. *elegans* RhoGTPase (CeCdc42) was tested using the AlphaScreen technology (PerkinElmer). Secondly, specificity of interaction between GAPs or GEFs and Rho GTPases was tested either by pull-down with glutathione-Sepharose beads and analyzed by western blot or using the AlphaScreen technology. ventional filtration assay with GST-fused GTPases loaded with [^{32}Pγ]-GTP. Thus far, our experiments suggested a high degree of specificity between GAPs and Rho GTPases. This paradigm is currently investigated at the structural level.

2 Introduction

Small GTP binding proteins are monomeric G proteins with a molecular mass of 20 to 40kDa. The family of Rho GTPases is implicated in many cellular processes including cell proliferation and cell mobility. Rho GTPases act like molecular switches that are turned ON and OFF in response to a variety of extracellular stimuli.

GTP-bound = ON conformation = interaction with effectors
GDP-bound = OFF conformation = no interaction with effectors

Three classes of proteins regulate this cycle:

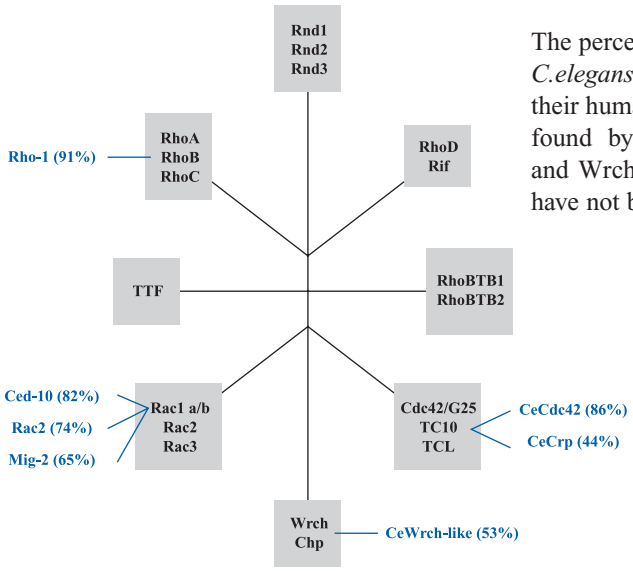
- GEFs: increase the exchange of GDP for GTP = ON state
- GAPs: increase the hydrolyse of GTP to GDP = OFF state
- GDIs: stabilise the GDP-bound form of the GTPase

Because of its flexibility and simplicity, C.elegans constitutes a powerful biological system to study the Rho GTPases regulatory mechanisms.

The high number of proteins and interactions that will be tested require a high-throughput system. Therefore, AlphaScreen technology (PerkinElmer) will be used to investigate:

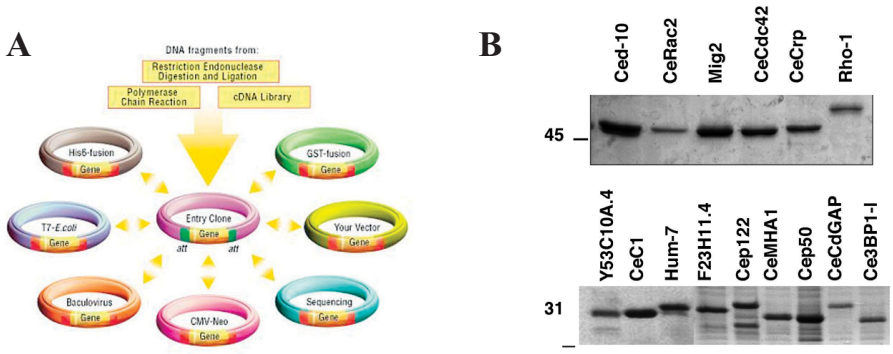
- Nucleotide binding on the GTPase (affinity for GTP/GDP)
- Interaction between GTPases and GAPs

3 *C.elegans* RhoGTPases and their human orthologs



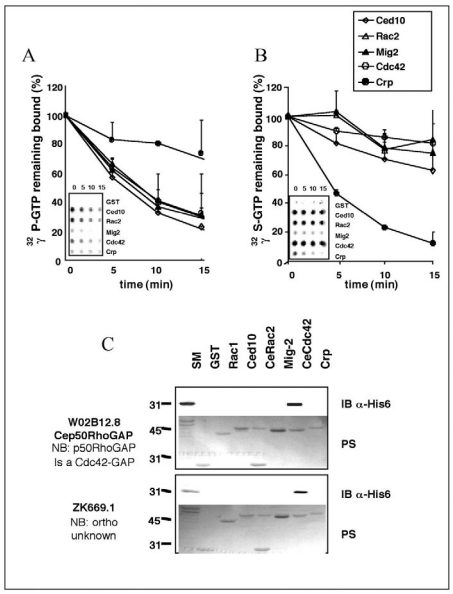
The percentage of homology between *C.elegans* Rho GTPases (blue) and their human orthologs (gray boxes) as found by sequence homology. Crp and Wrch-like are two GTPases that have not been characterized yet.

4 Cloning & expression of GST-CeRho GTPases and His-CeGAPs



Principle of GATEWAY™ technology (A). By recombination, cDNA of interest can be transferred from an entry vector to different expression vectors. Purified GST-CeRho GTPases and His-CeGAPs separated in SDS-PAGE and stained with Coomassie Blue (B).

5 *C.elegans* Rho GTPases display GTPase activity in vitro interact with GAPs



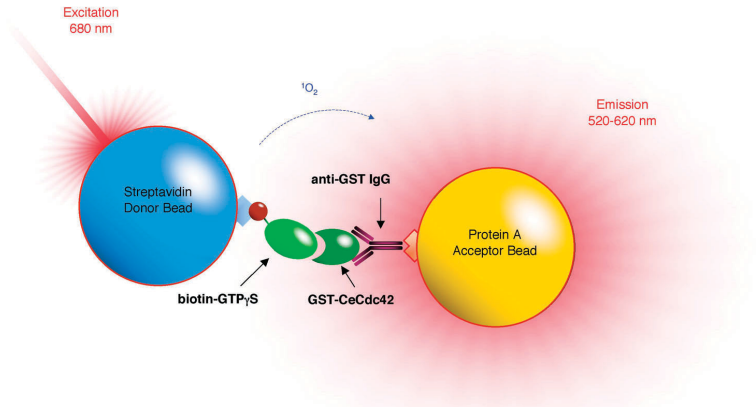
A) *In vitro* GTPase activity performed using conventional filtration assay with GST-fused GTPases loaded with [^{32}Pγ]-GTP.

B) *In vitro* GTPase activity using [^{35}Sγ]-GTP loaded GTPases.

C) GTPγ-S loaded GST-GTPases were incubated with bacterial lysates containing His-tagged GAPs and pulled-down with glutathione-Sepharose beads. Interacting GAPs are detected by western blotting using anti-His6 antiserum and GTPases visualized by ponceau staining.

6

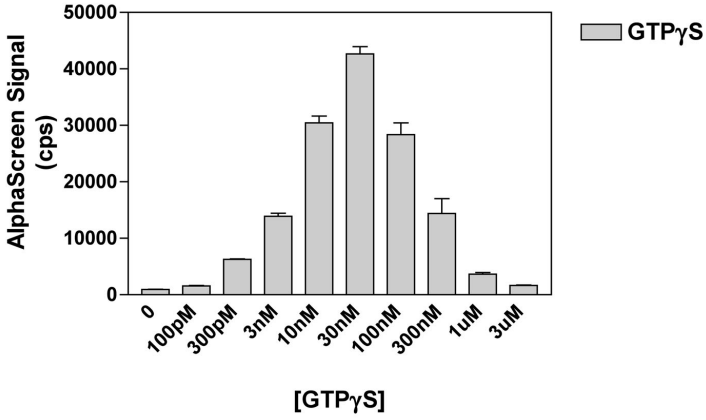
AlphaScreen assay setup



GST-fused RhoGTPases are detected by polyclonal anti-GST antibodies which are captured by Protein A coated Acceptor beads. Donor beads are used to capture biotin-GTPγS. Following the interaction between GST-GTPases and the biotin-GTPγS, AlphaScreen beads are brought into proximity. Singlet oxygen released from the Donor beads on laser excitation (680nm) reacts with fluorophores present within the Acceptor beads and an amplified signal is produced and measured at 520-602nm.

7

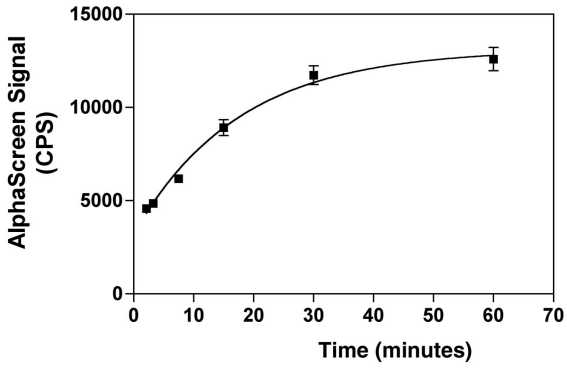
Optimization of AlphaScreen Assay conditions



Titration of biotin GTPγS. GST-CeCdc42 was incubated with different concentrations of biotin-GTPγS and 30nM of anti-GST antibody. After a short incubation, AlphaScreen beads were added in the wells. Signal was measured after 1 hour of incubation at 23ºC.

8

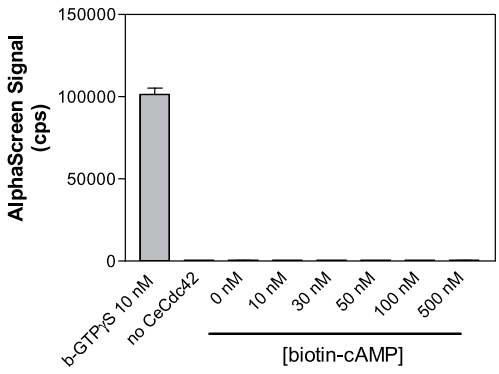
Time course of biotin-GTPγS binding to GST-CeCdc42



Time course of biotin-GTPγS binding to GST-CeCdc42. GST-CeCdc42 was incubated during different time intervals with 30nM of biotin-GTPγS and 30nM of anti-GST. The t1/2 value obtained is in agreement with value reported using other technologies (i.e. [^{35}S]GTPγS binding).

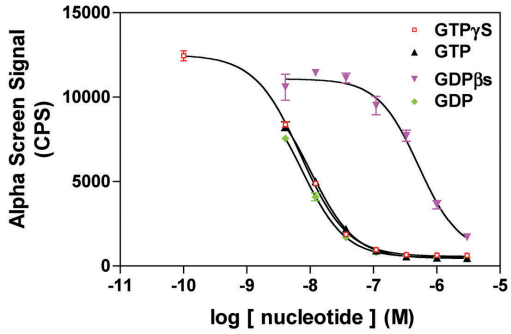
9

Specificity of biotin-GTPγS binding to GST-CeCdc42



Specificity of biotin-GTPγS binding to GST-CeCdc42. Different concentrations of biotin-cAMP were incubated with 30nM of GST-CeCdc42. As expected, no signal was generated by biotin-cAMP used as a negative control nucleotide. The presence of GST-CeCdc42 was also shown to be essential to produce the signal. These results, taken together, show the specificity of the biotin-GTPγS analog.

10 Relative affinity of various nucleotides for CeCdc42



Competition of GTPγS-biotin binding to Cdc42F+ by different nucleotides. Competition assay. GST-CeCdc42 (30 nM) was incubated in the presence of 30 nM of biotin-GTPγS and increasing concentrations of nucleotides. This assay provides relative affinity of nucleotides for Cdc42.

11 Conclusions

In the context of the C.elegans ORFeome project, consensus Rho GTPases and their regulators were identified and cloned using the GATEWAY™ technology (Invitrogen).

Our results show that:

- The recombinant CeRho GTPases are enzymatically active in vitro.
- The interaction between CeGAPs and CeRho GTPases is highly specific.
- AlphaScreen is an effective and specific tool to detect the interaction between nucleotides and RhoGTPases.

Preliminary results support the use of AlphaScreen™ for the development of a high-throughput system to analyse Rho GTPases interactions. All CeRho GTPases will be tested using the AlphaScreen nucleotide binding assay and the results obtained will be validated using conventional radioactive assays.

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