Regulation of Ras-Associated Protein-1 By Kinase Responsive to Stress B in *Dictyostelium discoideum*

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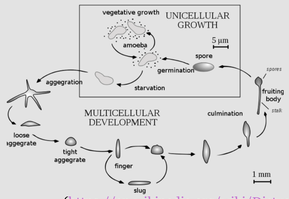
Abstract

Dictyostelium discoideum is a social amoeba that is commonly used as a model organism for studying chemotaxis, which is a directed migration along a chemical gradient, due to its similarities to human neutrophils and metastatic cancer cells. There are multiple pathways involved in regulating migration. In particular, kinase responsive to stress B (KrsB), a homolog of mammalian tumor suppressor MST1/2 and *Drosophila Hippo*, is a negative regulator of cell adhesion and migration in *D. discoideum*. However, little is known about the molecular mechanism of KrsB action. Another regulator of adhesion is small GTPase Ras-associated protein 1 (Rap1), which acts by affecting talin and myosin II. In mammalian cells Rap1 can be phosphorylated, which leads to its inhibition. We hypothesized that KrsB might negatively regulate Rap1 by phosphorylation, thereby disrupting the activation of Rap1 on the membrane. To determine if KrsB phosphorylates Rap1 we will perform immunoblotting for Rap1 in cells with or without KrsB and look for a shift in the electrophoretic mobility as an indicator of phosphorylation. In this study, we were able to detect RFP-tagged constitutively active Rap1^{G12V} on an immunoblot using an antibody against mCherry. We will now continue to conduct immunoblotting to detect mobility shifts of phosphorylated Rap1. To be able to track Rap1 localization, we successfully generated an RFP-Rap1 expression construct. We will examine RFP-Rap1 localization in cells with or without KrsB.

Introduction

- *Dictyostelium discoideum* is a social amoeba that has been used widely as a model organism.
- The life stages of *Dictyostelium discoideum* make it uniquely suited to study migration and chemotaxis – processes that contribute to human diseases such as cancer.[1]



(https://en.wikipedia.org/wiki/Dictyostelium_discoideum)

KrsB (Kinase Responsive to Stress B)

- KrsB is a homolog of tumor suppressors MST1/2 in mammalian cells and *Drosophila* Hippo.[1]
- KrsB plays a role in chemotaxis by being a negative regulator of cell adhesion and migration.[1]
- Cells lacking KrsB have increased contact with the surface and are more difficult to detach.[1]

Rap1 (Ras-Associated Protein-1)

- Rap1 is a small GTPase that is known to regulate adhesion in mammalian cells and in *Dictyostelium discoideum*.[2,4]
- In mammalian cells, Rap1 can be regulated by phosphorylation by cAMP-dependent protein kinase-A (PKA), and this phosphorylation negatively regulates Rap1.[2]
- Previous studies showed that Rap1 can regulate cell adhesion without KrsB, although KrsB might modulate Rap1 function.[4]

HYPOTHESIS

- **KrsB negatively regulates Rap1 by phosphorylation, which disrupts activation of Rap1 on the membrane.**

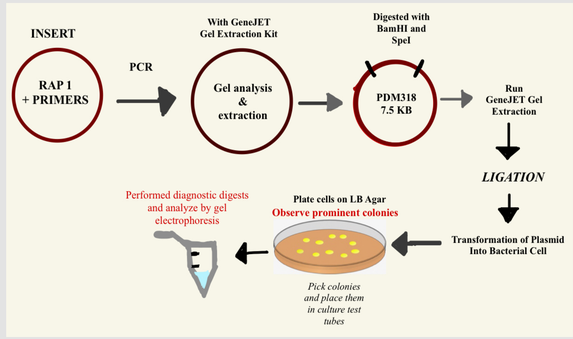
Approach

To look at the regulation of Rap1 by KrsB we plan to:

1) **Look at the localization of RFP-tagged Rap1 in the presence or absence of KrsB**
   - **This requires cloning of Rap1 into an expression plasmid with an RFP gene.**
2) **Look at the phosphorylation of Rap1 in the presence or absence of KrsB**
   - **To do this we need to examine if RFP-tagged Rap1 has an electrophoretic mobility shift indicative of phosphorylation in cells with KrsB compared to without.**
   - **To perform immunoblotting for RFP-tagged Rap1, first we need to test whether the mCherry antibody is able to recognize the RFP tag.**

Methodology

Cloning of RFP-Rap1



Results

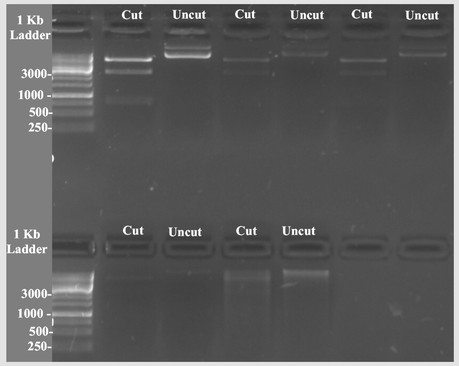


Figure 1. Diagnostic digest of plasmid with the Rap1 gene. Diagnostic digest was conducted for plasmid DNA isolated from bacterial colonies after transformation. The plasmid was digested with EcoRI and HindIII. The wells on the top panel showed the expected band sizes at around 4838 bp, 2411 bp, and 837 bp.

Results

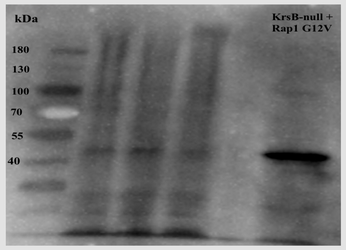


Figure 2. Detection of RFP-tagged constitutively active Rap1 (G12V) with an antibody against mCherry. Wild-type cells expressing RFP-Rap1 G12V were lysed, proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Membrane was immunoblotted with a primary antibody against mCherry, followed by a secondary antibody conjugated with horseradish peroxidase. Signal was detected by chemiluminescence. Signal was detected at the expected size (between 55 to 40 kDa).

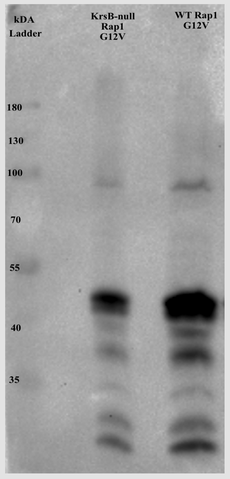


Figure 3. Detection of RFP-tagged constitutively active Rap1 (G12V) in KrsB-null and wild-type cells with an antibody against mCherry. Wild-type and KrsB-null cells expressing RFP-Rap1 G12V were lysed, proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Membrane was immunoblotted with a primary antibody against mCherry, followed by an anti-rabbit secondary antibody. Signal was detected by chemiluminescence.

Discussion

CLONING:

- PCR was successful for Rap1 and digest of both the insert and vector were successful. Gel extraction also proved to work for Rap1, and two different ligations were used to do transformation.
- Colonies from transformation were screened and gave the expected pattern in the diagnostic digest.
- Positive clones were confirmed by sequencing.

WESTERN BLOT:

- Immunoblotting showed that the antibody against mCherry can be used to detect RFP-tagged Rap1.
- Rap1 (G12V) in KrsB-null cell lysates and wild type cell lysates was detected on immunoblots, but did not show the electrophoretic shift under the current conditions.

Future Directions

- Analyze electrophoretic mobility of RFP-tagged Rap1 or Rap1 G12V in KrsB-null and wild type cells following stimulation of cells with a chemoattractant.
- Analyze Rap1–RFP localization in KrsB-null and wild-type cells by confocal microscopy.

References

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Acknowledgements

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