Spatial relationship between active RhoA and microtubules in the division plane during cytokinesis

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Abstract

Cytokinesis is the process in which a cell divides into two daughter cells. Before cytokinesis takes place, the cell needs to determine where the plane of division is located. How this division plane is placed is obscure. What is understood so far, is that the protein RhoA is localized at the periphery of the division plane and it assembles a contractile ring that pinches the cell into two. The plus-ends of microtubules from the opposing poles of the mitotic spindle intersect at the division plane. We hypothesize that the points of intersection of the microtubules contribute to positioning the RhoA proteins. We use *Xenopus* egg extracts and cells to test this hypothesis. To visualize active RhoA and microtubules in extracts and cells, we set out to clone, express, and purify two fluorescent recombinant proteins, GFP-Rhotekin and EMTB-mCherry. We have successfully expressed GFP-Rhotekin in E. coli and purified the protein. We have also completed cloning for the EMTB-mCherry. Next, we will introduce the proteins to extracts and cells to observe their colocalization during cytokinesis. The findings will enhance our knowledge in cytokinesis, and may further inform us on cell division-related diseases, such as cancer.

Introduction

Cytokinesis is the last step in cell division; during cytokinesis, a cleavage furrow forms on the cell's surface and cleaves the cell into two daughter cells. Active RhoA promotes cytokinesis by stimulating actin nucleation and myosin activation, thus, influencing the development of the cleavage furrow. Recent studies show that microtubule (MT) plus-ends activate RhoA.

Figure 1. Microtubules in *Xenopus* egg extracts under a confocal microscope.

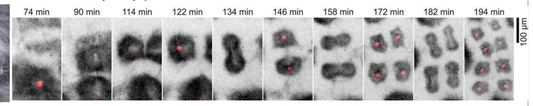

Figure 2. mCherry-NLS + BF cell-like compartments at different stages of mitosis as time continues with sperm in the cycling extracts to represent nuclei (ref. 1).

Xenopus egg extracts are a lysate made by spinning the eggs of the African clawed frog *Xenopus laevis*. When supplemented with sperm nuclei, extracts can form cell-like compartments that can undergo mitotic division to produce daughter compartments (Figure 2). They are optically clear and have been shown to recapitulate cytokinesis in *vitro*. Although *Xenopus* embryos are opaque, translucent blastomeres can be prepared from them (Figure 3). Thus, both egg extracts and translucent blastomeres allow direct observation of fluorescent RhoA and MTs.

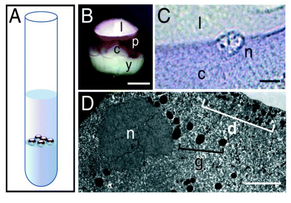

Figure 3. Preparation of translucent blastomeres. A) centrifugation. B) an embryo after centrifugation. C) micrograph showing a nucleus after centrifugation. D) electron micrograph showing a nucleus in centrifuged embryos. (ref. 2).

To fluorescently label RhoA and MTs, we utilize two proteins. Rho binding domain (RBD) of the human Rhotekin protein binds specifically to GTP-bound RhoA. Ensconsin protein (EMTB) is a conserved microtubule-associated protein (MAP) that interacts steadily with microtubules. We thus construct fluorescently tagged Rhotekin and Ensconsin to help visualize RhoA and MT. We will add the purified proteins to both the Xenopus egg extract and the Xenopus translucent blastomeres and apply fluorescence microscopy to observe the dynamics of active RhoA and MT plus-ends of kinetochore MT and astral MT in the division plane during cytokinesis.

Methods

Generation of expression plasmids: the gene encoding rhotekin (rGBD; Addgene plasmid # 26732) fused with GFP was cloned to a pGEX-based plasmid, which introduced a GST-tag at the N-terminus. The gene encoding ensconsin (EMTB; GeneBank ID: X73882; Addgene plasmid # 26742) fused with 2x mCherry (N terminal on insert) was cloned to a pGEX-based plasmid and fused with a GST-tag.

DNA Cloning: The backbone and insert go through PCR (Polymerase Chain Reaction); it is followed by PCR purification and gel electrophoresis. If the gel electrophoresis results are satisfactory, we perform ligation so that the insert and the backbone are connected. Next, we perform transformation, which is the process of DNA plasmids being transferred into bacteria in order to express the DNA in the bacteria. We then perform miniprep, a process that extracts the DNA from the bacteria. The DNA sequence is then further verified by enzyme digestion and DNA sequencing.

Protein Expression: the process in which genes are translated into proteins. We used IPTG to induce protein expression. Bacteria cultures are grown with IPTG at 16°C and then harvested. The proteins are then purified. During purification, bacteria are first harvested by centrifugation. Then, they are resuspended and lysed by a sonicator. The lysed bacteria are centrifuged and the supernatant is filtered. The supernatant was incubated with glutathione beads in an affinity chromatography column, and then eluted by reduced glutathione. The protein fractions were concentrated by a protein concentrator column. Purified proteins and different fractions from the purification process are analyzed by SDS-PAGE using polyacrylamide gel and coomassie stain.

Results

pGEV-GFP-Rhotekin

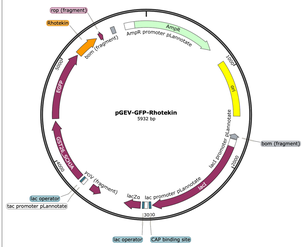

Figure 4. pGEV-GFP-Rhotekin The gene encoding rhotekin (rGBD; Addgene plasmid # 26732) together with GFP (C terminal on backbone) was cloned to pGEX-based plasmid and fused with a GST-tag.

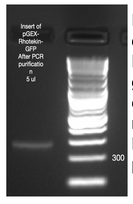

Figure 5. insert of pGEV-GFP-Rhotekin DNA gel electrophoresis result after PCR purification.

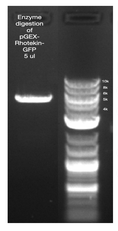

Figure 6. pGEV-GFP-Rhotekin DNA gel electrophoresis result after enzyme digestion with band size of 5k.

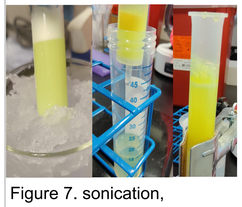

Figure 7. sonication, filtration, and elution of proteins during protein purification.

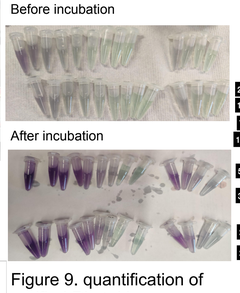

Figure 9. quantification of purified Rhotekin concentration. Row 1 and Row 2 represent samples before incubation. Row 3 and Row 4 represent samples after incubation in a water bath at 37°C for 30 minutes.

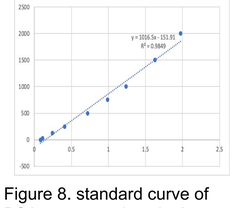

Figure 8. standard curve of BSA concentrations in the BCA assay for quantifying protein concentration (mg/ml).

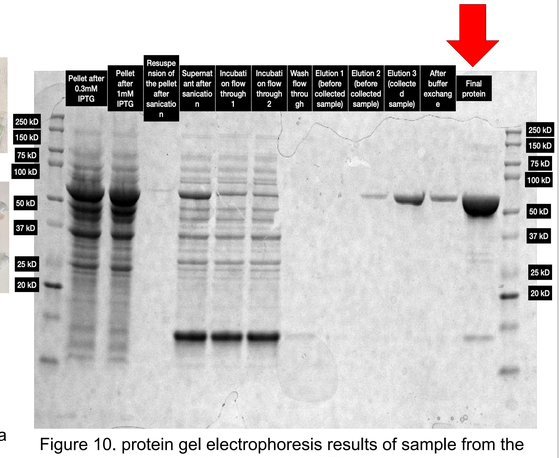

Figure 10. protein gel electrophoresis results of sample from the different stages of protein purification. The thick band in the land indicated by the red arrow is the desired purified GST-GFP-Rhotekin.

Results

pGEX-EMTB-mCherry

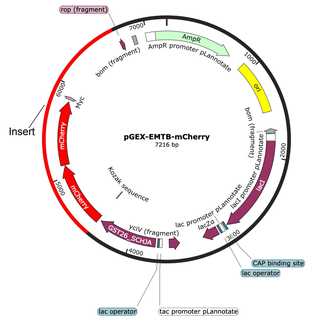

Figure 10. pGEX-EMTB-mCherry The gene encoding ensconsin (EMTB; GeneBank ID: X73882; Addgene plasmid # 26742) together with 2x mCherry (N terminal on insert) was cloned to pGEX-based plasmid and fused with a GST-tag.

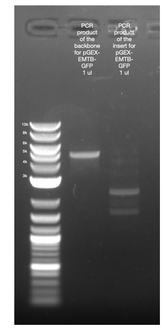

Figure 11. pGEX-EMTB-mCherry backbone (band size 5k) and insert (band size unclear) DNA gel electrophoresis results after PCR.

Summary

To study the mechanism of cytokinesis, we used recombinant probes to visualize two key regulators, RhoA and microtubules. The first probe is GFP-Rhotekin, which visualizes RhoA. The second probe is EMTB-mCherry, which visualizes microtubules. These probes can be used in both egg extracts and injected into cells. In this work, plasmid pGEV-GFP-Rhotekin is successfully purified and EMTB-mCherry has been successfully cloned. Production of GFP-Rhotekin overnight at 16C in *E. coli* was found to facilitate high levels of these proteins in the cell lysate. Rhotekin was efficiently purified using a glutathione affinity chromatography column with high yield; in total, we obtained 3 ml of highly purified protein solution, with a concentration of 3000 ug/ml from 1 L bacterial culture.

Future work will focus on expressing Ensconsin-mCherry protein and introducing both proteins in *Xenopus* egg extract and the *Xenopus* translucent blastomeres to reveal the position relationship between active RhoA and microtubule plus-ends in the division plane.

Sources

1. Cheng X, Ferrell JE Jr. Spontaneous emergence of cell-like organization in Xenopus egg extracts. Science. 2019 Nov 1;366(6465):631-637. doi: 10.1126/science.aav7793. PMID: 31672897; PMCID: PMC7839252.
2. Iwao, Y., Uchida, Y., Ueno, S., Yoshizaki, N. and Masui, Y. (2005), Midblastula transition (MBT) of the cell cycles in the yolk and pigment granule-free translucent blastomeres obtained from centrifuged Xenopus embryos. Development, Growth & Differentiation, 47: 283-294. https://doi.org/10.1111/j.1440-169X.2005.00802.x

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