Phosphorylation Regulates Myosin Driven Organelle Movements

Peter Duden and Andreas Nebenführ
Department of Biochemistry and Cellular and Molecular Biology, University of Tennessee, Knoxville, 37919

Abstract

Cytoplasmic streaming in plant cells is the continuous flow of cytoplasm and organelles throughout the cell, with the first observation of cytoplasmic streaming being published in 1774.[1] The root hairs of *Arabidopsis thaliana* provide excellent cells to study this phenomenon due to their rapid polarized growth at the root hair tip (Figure 1). Experimentation with this model system and animal based models has demonstrated that the cytoskeleton is a main mechanistic component of cytoplasmic streaming. Research now supports that the motive force generating cytoplasmic streaming in plants may be through the interaction of myosin XI motor proteins with organelles while sliding along actin filaments.[2, 3, 4] From this, a key topic of interest is how myosin driven organelle movement is regulated. Our research focuses on whether phosphorylation affects the regulation of myosin XI motor proteins. Specifically, the goal of our research is to determine the presence of regulation of myosin XI motors by phosphorylation, and whether phosphorylation has a significant effect on cytoplasmic streaming.

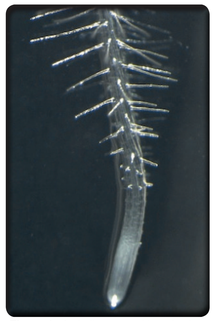


Figure 1. *A. thaliana* root with projecting root hairs demonstrates active polarized growth.

The presence of phosphorylation regulation on myosin XI trafficking was determined with kinase and phosphatase inhibitors on myosin and organelle localization and movement in Arabidopsis thaliana. We observed significant changes in the velocities of myosin isoform MYA1 and fluorescently tagged organelles after addition of inhibitors. The number of unique tracks also changed after inhibition, indicating increased cytoskeleton remodeling. The localization of organelles and the myosin isoform remained the before and after addition of inhibitors. Our results support the conclusion that phosphorylation is a regulator of myosin driven organelle movement. The change in velocities caused by phosphorylation appears to vary among different types of organelles.

Introduction

Cytoplasmic streaming is a motive force throughout the cell that distributes organelles, metabolites, and cytoplasm. Although cytosol movement is present in both plant and animal cells, the cytoplasmic streaming in the former is more prominent as a result of an inability to change shape or position from their cell wall.[2] Essential to cell viability, a significant amount of research is aimed to discover its mechanistic basis. The root hairs of *A. thaliana* provide an excellent model to study cytoplasmic streaming, as they are unicellular, require significant cytoplasmic streaming as a result of fast polarized growth (greater than 1 µm/s), and are positioned away from the main root for clear observation.

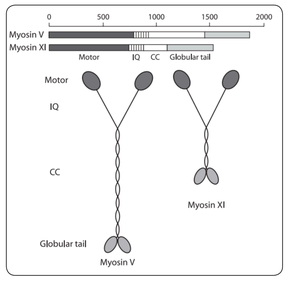


Figure 2. Myosin motor proteins are most conserved in the motor domain with differentiation at the globular tail. Myosin V, well characterized in mammalian cells, is similar to myosin XI. Figure: Li and Nebenführ, 2008.

When cytosol from plant cells was observed to be insufficient in generating cytoplasmic streaming alone, a molecular motor was hyporthesized as the possible source.[4] Myosins, known as ATP-dependent motor proteins, are now most supported to be this motor. Myosin motors are in close interaction with actin filaments, which function as tracks longitudinally across the cell. Of the 35 know classes, VIII and XI myosins are found in plants and play active roles in cytoplasmic streaming. Support of these acto-myosin systems in cytoplasmic streaming includes research that inhibition of plant myosin in pollen tubes results in halted cytoplasmic streaming, and that isolated myosin motor proteins move at the same velocity as cytoplasmic streaming in identical cell types.[6, 7]

Myosin XI is the fastest known myosin motor, processively moving along actin at 7 µm/s due to its high rate of ATP hydrolysis and large displacement in each step.[8] As such, myosin XI is optimal for carrying organelles across a growing cell, with many organelle interactions being discovered in recent research.[9] Current research in our lab focuses on the regulation of myosin XI and its organelle cargo in relation to cytoplasmic streaming. Regulation of class V myosin in mammals, which has many conserved stuctures in comparison to class XI myosins, is facilitated by phosphorylation (Figure 2). Previous research has shown that Golgi bodies are affected in movement by phoshatase in-hibitors, while actin filaments do not become destroyed or disrupted (Nebenführ, 1999). In this experiment, two inhibitors of phosporylation were utilized in order to observe the regulatory role phosphorylation has on organelle velocities caried by myosin XI. The kinase inhibitor staurosporin and the phosphatase inhibitor okadaic acid were used to determine the effects of phosphosphorylation.

Acknowledgements and References

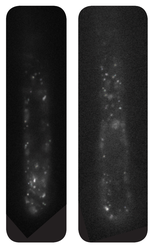
I thank Dr. Nebenführ for his guidance during the REU program and throughout my undergraduate education. I also show my appreciation to Eunsook Park for providing her seed lines to the lab. The National Science Foundation has my gratitude for funding of the REU grant.

1. Allen R, Allen N. (1978). Cytoplasmic streaming in ameboid movement. Annu Rev Biophys Bioeng 7:469-495.
2. Shimmen T. (2007). The sliding theory of cytoplasmic streaming: Fifty years of progress. J. Plant Res. 120: 31-43.
3. Higashi-Fujimi, S., Ishikawa, R., Iwasawa, H., Kagami, O., Kurimoto, E., Kohama, K., Hozumi, T. (1995). The fastest actin-based motor protein from the green algae, Chara, and its distinct mode of interaction with actin. FEBS Lett. 375:151-154.
4. Kamiya, N., Kuroda, K. (1956). Velocity distribution of the protoplasmic streaming in Nitella cells. Bot. Mag. 69: 544-54.
5. Sellers, J.R. (2000). Myosins: a diverse superfamily. BBA 1496, 3-22.
6. Tominaga, M., Yokota, E., Sonobe, S., and Shimmen, T. (2000). Mechanism of inhibition of cytoplasmic streaming by a myosin inhibitor, 2,3- butanedione monoxime. Protoplasma 213, 46- 54.
7. Peremyslov V.V., Prokhnevsky A.I., Dolja V.V. (2010). Class XI myosins are required for development, cell expansion, and F-Actin organization in Arabidopsis. Plant Cell 22, 1883–1897.
8. Tominaga M., Kojima H., Yokota E. (2003). Higher plant myosin XI moves processively on actin with 35 nm steps at high velocity. EMBO J. 22, 1263– 1272.
9. Madison, S.L., and Nebenführ, A. (2013). Understanding myosin functions in plants: are we there yet? Current Opinion in Plant Biology 16, 710-717.

Central Questions

How does phosphorylation affect myosin XI localization and velocity?
Does the effect of phosphorylation differ among organelles?

MYA1



Myosin MYA1 localization was observed as most concentrated at the root hair tip. MYA1 signal increased slightly at shank of root hair after inhibitor treament. MYA1 velocities decreased after okadaic acid treaments, and increased after staurosporin treatments (One way ANOVA, p-value < 0.01).

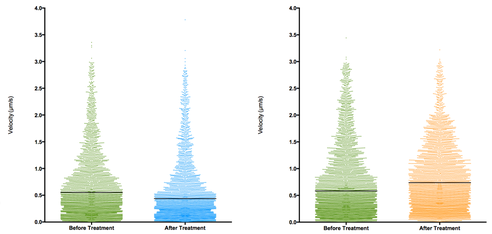
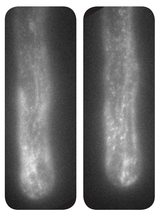


Figure 5. Changes in velocities of MYA1 from okadaic acid (left) and staurosporin (right).

RabA4b



RabA4b vesicle localization did not significantly change after inhibitor treatments, with only a small increase in signal at the shank of the root hair. RabA4b velocities although significant, were marginally affected by either okadaic acid or staurosporin (One way ANOVA, p-value < 0.01).

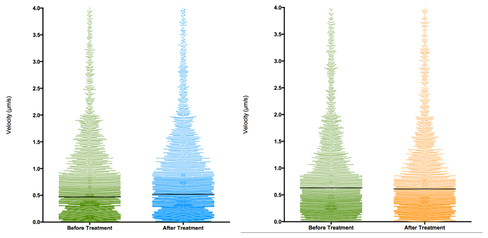
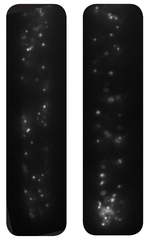


Figure 6. Changes in velocities of RabA4b from okadaic acid (left) and staurosporin (right).

Peroxisomes



Peroxisome signal velocities increased after administration of okadaic acid, and were only marginally affected by staurosporin (One way ANOVA, p-value < 0.01). Localization spread throughout the root hair, with a small increase in signal size after inhibitor treatment.

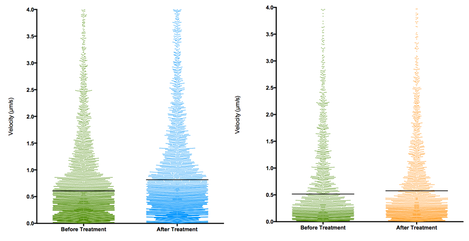
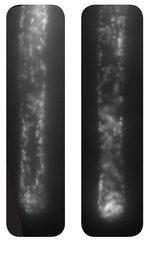


Figure 7. Changes in velocities of peroxisomes from okadaic acid (left) and staurosporin (right).

Golgi Bodies



Golgi bodies were localized throughout the root hair in close proximity and number. Localization was not affected by inhibitors. Velocities increased after administration of okadaic acid, and decreased slightly with staurosporin (One way ANOVA, p-value < 0.01).

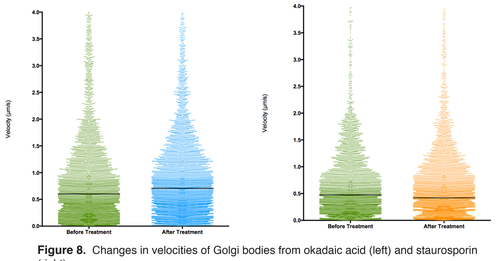


Figure 8. Changes in velocities of Golgi bodies from okadaic acid (left) and staurosporin (right).

Methods

Plant Lines and Seed Preparation

Three *A. thaliana* seed lines were used for this experiment. All single YFP and CFP markers for MYA1 and RabA4b seed lines were created in our lab previously. A triple organelle marker (TOM) was also utilized, containing peroxisome-CFP, Mitochondria-YFP, and Goldi-mCherry in one binary vector. TOM was created through combining Golgi-mCherry with Mitochondria-YFP and Peroxisome-CFP by individual 35S promoters in binary vector pFGC19. Both the single and triple organelle markers were transformed with *Agrobacterium tumefaciens*. For growth conditions, RabA4b, MYA1, and TOM seedlings were placed on vertical plates (1/4 Murashige - Skoog) and incubated for 5 days.

Fluorescence Microscopy

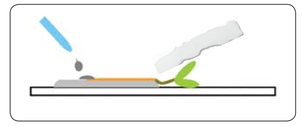


Figure 3. Apparatus to administer inhibitor in root hairs. Care was taken to not touch the seedling by only dropping inhibitor on the cover slip. Inhibitor was given 10 minutes to take incubate. Figure: Wu et al., 2012.

Fluorescence microscopy was then utilized in order to observe the signals from each of the grown seedlings. A cover slip was pasted with vacuum grease on two sides and gently placed over the seedling to create an observation chamber, which was filled with medium. A one minute timelapse with one picture per second was taken, in addition to pictures of five different cross sections of the root hair to observe localization. After this, three aliquots of 100 µL of dimethyl sulfoxide (DMSO; 1% in MSNS), staurosporin (200 nM in MSNS and 1% DMSO), or okadaic acid (200 nM in MSNS and 1% DMSO) replaced the original medium. The medium was removed by allowing a piece of paper to contact the observation chamber on the opposite side of where the inhibitor or control is being added, allowing the medium to enter the paper as the inhibitor pushes the medium (Figure 3). After 10 minutes, data collection was repeated.

Statistical analysis of MYA1, RabA4b, and TOM

Organelle signals in timelapses were tracked through an automated particle tracker in ImageJ. Parameters were adjusted until the tracker identified most signals correctly with the least amount of tracks being reported (Figure 4). A final macro was run to visualize remaining errors and to provide data so that the total percentage of incorrect tracks could be calculated.The optimized data contains the velocities and number ofunique tracks present in each root hair. A minimum of 4 root hairs for each signal were obtained for analysis.

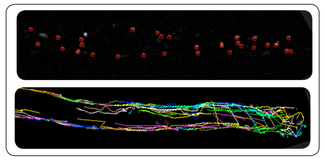


Figure 4. Automated particle tracking in ImageJ. Particles are identified through red circles based on inputted paramters. Once the automation is completed, data is outputted as well as an colored image of all tracks.

Conclusions

1. Phosphorylation decreases the movement of MYA1 and dephosphorylation increases the movement of MYA1.
2. RabA4b movement is not regulated by phosphorylation as it is not affected by treatment with inhibitors.
3. Peroxisomes have increased movement when phosphorylated.
4. Golgi bodies have decreased movement when unphosphorylated and increased movement from phosphorylation.

From these conclusions, phosphorylation is supported to regulate myosin XI and its cargo in trafficking.

Further Research

- Continue optimizing particle tracking analysis to eliminate remaining errors in data
- Determine novel approaches in tracking signals that are too diffuse or numerous with current macro (Endoplasmic Reticulum, Trans Golgi Network, Mitochondria)
- Determine the proportion of signals actively streaming against stationary signals, and develop coding to filter stationary signals accurately
- Utilization of other kinase/phosphatase inhibitors to observe if effect is replicated
- Control studies using actin polymerization inhibitor (latruncilin) to account for actin dynamics
- Observe growth rate of root hairs under continued phosporylated/unphosporylated myosin XI

ABSTRACT

Cytoplasmic streaming in plant cells is the continuous flow of cytoplasm and organelles throughout the cell, with the first observation of cytoplasmic streaming being published in 1774. However, the mechanism of cytoplasmic streaming remained unclear until components of the cytoskeleton were researched. Research now supports that the motive force generating cytoplasmic streaming is the interaction of myosin XI motor proteins with organelles while sliding along actin filaments. From this, a key topic of interest is how myosin driven organelle movement is regulated. Our research focuses on whether phosphorylation affects the regulation of myosin XI motor proteins. Specifically, the goal of our research is to determine the presence of regulation of myosin XI motors by phosphorylation, and whether phosphorylation has a significant effect on cytoplasmic streaming.

The presence of phosphorylation regulation on myosin XI trafficking was determined with kinase and phosphatase inhibitors on myosin and organelle localization and movement in *Arabidopsis thaliana*. We observed significant changes in the velocities of myosin isoform MYA1 and fluorescently tagged organelles after addition of inhibitors. Our results support the conclusion that phosphorylation is a regulator of myosin driven organelle movement. Recent research suggests the change in velocities caused by phosphorylation varies among different types of organelles.