Proceedings of The National Conference
On Undergraduate Research (NCUR) 2013
University of Wisconsin La Crosse, WI
April 11 – 13, 2013

Phosphorylation Levels of the Inner-Dynein Arm Subunit, IC138, are altered in Response to a High Calcium Treatment

Todd A. Jackson
Department of Biology
Morehouse College
Atlanta, GA

Faculty Advisor: Dr. T. W. Hendrickson

Abstract

Cilia and flagella are extensions of the plasma membrane and are ubiquitously present in organisms of the Eukaryotic kingdom. The high incidence of these cellular appendages and the conservation of flagellar and ciliary genes among organisms can be attributed to their significant roles. Between these two structures, functions in motility, homeostasis, reproduction, physical defense and cell proliferation are recognized. Ciliary and flagellar motility is regulated by changes in intraflagellar calcium. Calcium activates signaling pathways that lead to cellular responses. The biflagellate alga, *Chlamydomonas reinhardtii* is a powerful model system used to study the ciliary biology. The 3-dimensional configuration of dynein motors, which have salient roles in the power-stroke and waveform of cilia and flagella, are of interest, particularly that of I1. I1 is an inner arm dynein motor complex composed of two heavy chains (1α-HC and 1β-HC), three intermediate chains (IC138, IC140 and IC97), and several light chains. Phosphorylation of the IC138 subunit of the inner dynein arm I1 has been shown to regulate the activity of the dynein complex and thus motility. To test the hypothesis that calcium regulation of dynein activity is occurring via IC138, the levels of IC138 were measured using a mobility shift assay. The results indicate that the phosphorylation state of IC138 is altered in response to treatment with a high calcium buffer.

Keywords: Dynein, Phosphorylation, Calcium

1. Introduction

Cilia and flagella are important structural components of eukaryotic cells. While both cilia and flagella are able to move liquid in a whip-like fashion across the cell surface, cilia are unique in that there are two types: motile and immotile. As in the name, motile cilia mediate movement of the cell or fluid around the cell, while immotile cilia serve a sensory function necessary in cell division, cell polarity, and act as chemo-, mechano-, and photo-receptor sensors. Most eukaryotic cells contain one immotile cilium; these single immotile cilia are referred to as primary cilia.

Cilia and flagella are structurally similar and will from here on be referred to as cilia. The basic structure of a cilium is the microtubule-based axoneme, which originates from the basal bodies. The axoneme is composed of nine outer doublet microtubules, inner and outer dynein arms, connecting nexin links, radial spokes, a pair of singlet microtubules and other associated proteins [1,2,3]. When viewed longitudinally, the arrangement of the axonemal dyneins repeat every 96 nm along the doublet microtubule [3]. In motile cilia, the nine outer doublet microtubules encircle the two singlet microtubules, the central pair (9+2), while in immotile cilia the central pair apparatus is absent (9+0).

The dynein motor proteins, located on the outer doublet microtubules, generate the force required for motility. The dyneins are arranged into complexes and characterized by their position on the doublets. The outer dynein arms

are responsible for most of the force that drives motility while the inner dynein arms serve mostly in a regulatory capacity [4,5,6].

The inner dynein arm complex that is most studied and attributed for the regulation of dynein activity is I1. The I1 inner dynein arm complex is composed of two heavy chains (1□HC, 1□HC), three intermediate chains (IC140, IC138, IC97), and several light chains (LC7a, LC7b, TcTex1 and TcTex2b). Previous studies have shown that regulation of dynein activity occurs primarily through phosphorylation/dephosphorylation of the IC138 dynein [7,8,9,10,11,12]. Furthermore, treatment with calcium has also been shown to regulate dynein activity in radial spoke (RS) and central pair (CP) mutants: *pf*15, *pf*16, *pf*17, and *pf*18 [13]. This implies a regulatory pathway wherein the central pair apparatus and radial spokes modulate motility by regulating dynein activity (see Figure 1); however, the role of calcium as it relates to localized regulation of the molecular motors responsible for motility, the dynein arms, has not been fully elucidated. Our working hypothesis is that IC138 is regulated by axonemal kinases and phosphatases, which are in turn, regulated by calcium. To determine whether calcium regulates dynein activity through the I1 IC138, we assessed the phosphorylation levels of IC138 and doublet microtubule sliding velocity in response to calcium treatment.

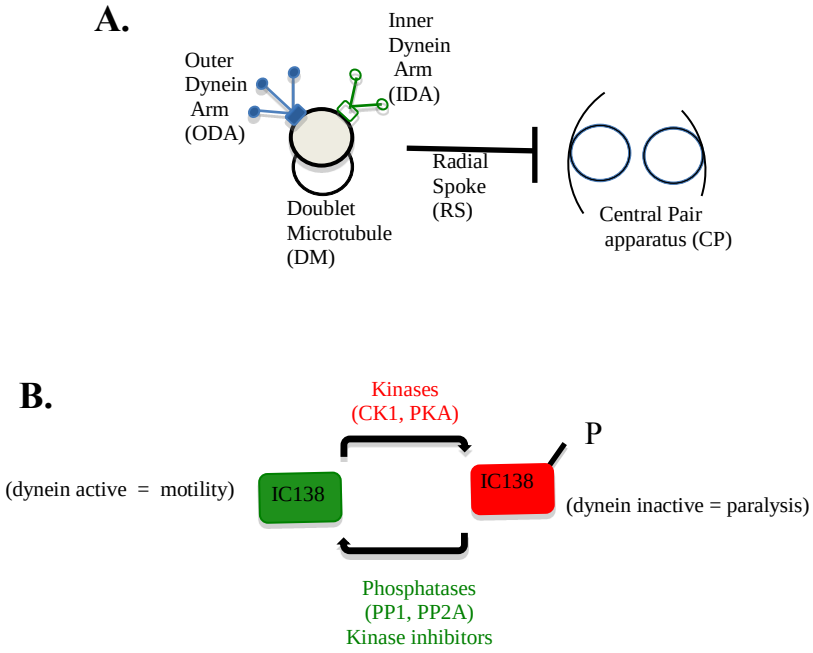

Figure 1. (A) Arrangement of the dynein complexes relative to the radial spokes and central pair apparatus. (B) The phosphorylation state of IC138m an intermediate chain dynein of the I1 inner dynein arm regulates the activity of the dynein complex and thus ciliary motility.

2. Methodology

2.1 Cell Strains and Growth Conditions

The *Chlamydomonas reinhardtii* strains used in this study were wild type (CC-124), *pf18* (CC-1036, missing central pair apparatus), *pf17* (CC1035, missing radial spoke head), and *pf14* (CC-613, missing radial spokes). All strains were obtained from the Chlamydomonas Resource Center. Cells were grown in TAP media with aeration for 3 days on a 14/10-light/dark cycle at room temperature. On the day of axonemal protein isolation, one half of the culture was treated with $CaCl_2$ while the other half was left untreated.

2.2 Isolation of Axonemes and CIP treatment

Axonemes were isolated as previously described by Witman [14]. Briefly, cells were pelleted and resuspended in HMDS (10mM HEPES, pH 7.4, 5 mM MgSO4, 1mM DTT, 4% Sucrose (w/v)). The flagella were separated from the cell body using Dibucaine in HMDEgS (10mM HEPES, pH 7.4, 5mM MgSO4, 1mM DTT, 0.5mM EGTA, 4% Sucrose (w/v)). Following demembranation by a nonionic detergent (0.5% NP-40), the axonemes were resuspended in HMDENa (10mM HEPES, pH 7.4, 5 mM MgSO4, 1mM DTT, 0.5 mM EDTA, and 30 mM NaCl). For the Calf Intestinal Phosphatase (CIP) experiment, axonemes were treated with 1mM CIP for 1 hour at room temperature then processed as gel samples. All other samples were processed as gel samples directly after resuspension in HMDENa.

2.3 Gel Electrophoresis and Western Blotting

Following protein isolation, the axonemes were separated by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% Tris-HCl gels (Bio-Rad). Western blotting was done to determine the phosphorylation state of IC138 using the following antibodies: α-IC138 (1:10,000); α-IC140 (1:10,000); Goat-anti-rabbit HRP (1:10,000, Bio-Rad).

2.4 Motility Assay

Microtubule sliding velocity was measured as previously described [15]. Briefly, flagella were isolated and resuspended in HMDEK ((10mM HEPES, pH 7.4, 5 mM MgSO4, 1mM DTT, 0.5 mM EDTA, and 30 mM KCl) without protease inhibitor. Following demembranation with NP-40 as previously described above, the axonemes were added to a perfusion chamber, and then washed with HMDEK containing 1mM ATP. To initiate microtubule sliding a Serine endoproteinase (Type VIII, Sigma-Aldrich P-5380) was added to the HMDEK-ATP buffer. Microtubule sliding was visualized by dark field microscopy using a Zeiss Image A2 microscope equipped with an AxioCam MRc. The images were analyzed using the Zen software. The average microtubule sliding velocity was calculated from three different experiments, each with a sample size of at least 10 axonemes.

3. Results

IC138, one of the intermediate chains of the I1 inner dynein arm complex, is a key regulatory phosphoprotein. IC138, along with IC97, LC7b and FAP120 comprise the IC138 subregulatory complex, which is required for maintaining proper waveform [16]. To establish the phosphorylation levels of IC138 for the purpose of this study, calf intestinal phosphatase (CIP) was used to treat axonemes isolated from wild type cells, central pair mutants (*pf18*) and radial spoke mutants (*pf17*). As previously shown by Hendrickson *et al.* [15]the central pair and radial spoke mutants contain elevated levels of phosphorylated IC138, as seen by a mobility shift in IC138 after gel electrophoresis (Figure 2). To determine whether calcium is responsible for the altered the phosphorylation state of IC138, cells were grown in the absence or presence of high calcium (10^{-4} M). Axonemes were isolated, separated by SDS-PAGE then visualized by Western blotting. As shown in Figure 3, there is less phosphorylation, as seen by band smearing, of IC138 in axonemes isolated from wild type cells compared to that of the central pair (*pf18*) and radial spoke (*pf14*) mutants. This suggests that high calcium levels lead to a decrease in the phosphorylation of IC138.

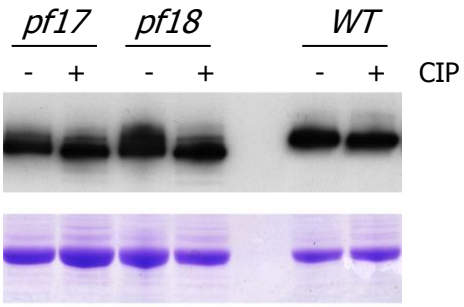

Figure 2, IC138 is hyperphosphorylated in paralyzed flagella mutants. Axonemes were isolated from central pair (pf18) and radial spoke (pf17) mutants. Following isolation the axonemes were treated with CIP then analyzed by SDS_PAGE and Western Blotting for IC138. CIP treatment of axonemes isolated from central pair (*pf18*) and radial spoke (*pf17*) mutants reveal a pattern of hyper-phosphorylation of IC138 that is not seen in wild type as previously reported by Hendrickson *et al*., 2004.

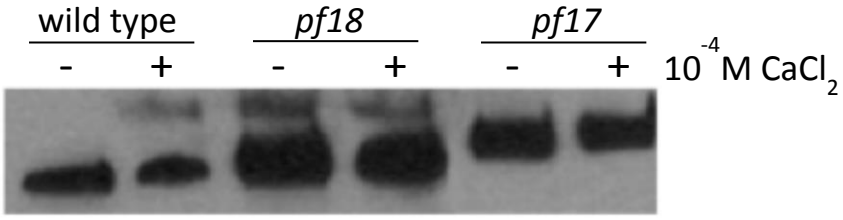

Figure 3. Calcium decreases IC138 phosphorylation in *pf* mutants. IC138. Axonemes isolated from WT and CP (*pf18*) and RS (*pf14*) mutant cells grown in the presence or absence of 10^{-4} M Ca^{2+} were subjected to Western blotting to visualize the phosphorylation pattern of IC138 in response to calcium treatment

To determine whether the decrease in IC138 phosphorylation seen on by Western blotting results in a physiological effect the on dynein activity, microtubule sliding velocities of wild type and radial spoke mutant cells were tested in the presence and absence of 10^{-4}M $CaCl_2$. Doublet microtubule sliding can be initiated by the removal of the nexin links, which tether the doublets to each other in the axoneme. Microtubule sliding results from the dynein arms on a doublet "walking" along the adjacent doublet microtubule in the minus direction. Thus, microtubule sliding velocity can be used as a direct measure of dynein activity. Treating the cells with calcium prior to the microtubule sliding assay resulted in an increase in sliding velocity for both the wild type and the mutant cells (Figure 4).

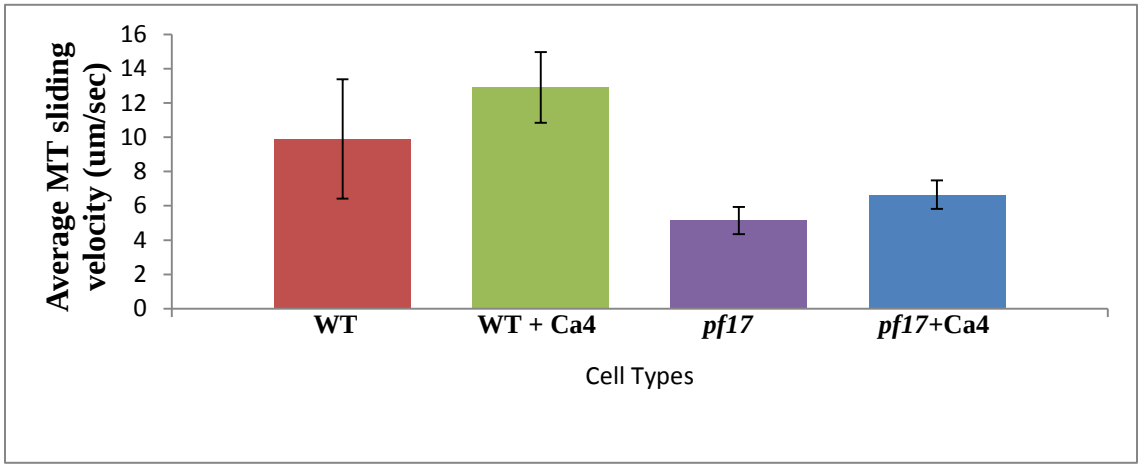

Figure 4. Calcium increases microtubule sliding velocity in axonemes isolated from cells treated with 10^{-4}M $CaCl_2$. Cells were treated with calcium as described above then deflagellated. The flagella were demembranated and subjected to a microtubule sliding assay. The average velocity for each cell type is reported here.

4. Discussion

The objective of this study was to determine whether calcium alters the phosphorylation state of IC138. Our working hypothesis was that IC138 is regulated by axonemal kinases and phosphatases, which are in turn, regulated by calcium. The data reported here show that calcium treatment leads to a decrease of IC138 phosphorylation. Exposure to elevated levels of calcium show phosphorylation patterns in treated groups mimicking general phosphotase activity. Additionally, motility increases in wild type and mutants when exposed to higher calcium concentrations.

IC138 is a phosphoprotein in the I1 inner dynein arm complex. It is essential for the assembly of the IC138 regulatory subcomplex, comprised of IC138, IC97, LC7b and FAP120 [16]. In the absence of a complete IC138 regulatory subcomplex, dynein activity is altered, which is manifested in altered motility and waveform [16,17]. IC138 is regulated by a network of kinases and phosphatases, some of which are associated with the radial spokes [7, 8, 9, 10, 12]. The prevailing hypothesis that these kinases phosphorylate IC138, rendering it inactive [4,7,12]. In the absence of the kinases, or the presence of phosphatases, the IC138 is activated and can once again regulate motor activity. Furthermore, in the absence of the radial spokes, the IC138 appears to be locked in the inhibited state, as is seen by increased phosphorylation and the decreased microtubule sliding velocity. The absence of the central pair apparatus also results in a constitutively inhibited IC138. However, addition of calcium seems to relieve this inhibition and restore IC138 to its active state.

The next step will be to compare the calcium-mediated inhibition release to that of kinase inhibitors to determine which kinases are being affected by the calcium; presumably, the calmodulin-binding kinase (CaM Kinase) will be the most affected. This will provide much needed insight to the regulation of motor activity by calcium.

5. Acknowledgements

The research reported in this publication was supported by the National Institute of General Medical Science of the National Institutes of Health MBRS-SCORE award to TWH (5S06G067078-02). TAJ was supported by the National Institutes of Health MBRS-RISE program at Morehouse College (5R25GM070514).

6. References

[1] D. Nicastro, C. Schwartz, J. Pierson, R. Gaudette, M.E. Porter, J.R. McIntosh, "The molecular architecture of axonemes revealed by cryoelectron tomography", Science 313 (2006) 944-948.

[2] T. Heuser, C.F. Barber, J. Lin, J. Krell, M. Rebesco, M.E. Porter, D. Nicastro, "Cryoelectron tomography reveals doublet-specific structures and unique interactions in the I1 dynein", Proc Natl Acad Sci U S A (2012).

[3] D.N. Mastronarde, E.T. O'Toole, K.L. McDonald, J.R. McIntosh, M.E. Porter, "Arrangement of inner dynein arms in wild-type and mutant flagella of Chlamydomonas", J Cell Biol 118 (1992) 1145-1162.

[4] M. Wirschell, T. Hendrickson, W.S. Sale, "Keeping an eye on I1: I1 dynein as a model for flagellar dynein assembly and regulation", Cell Motil Cytoskeleton 64 (2007) 569-579.

[5] H. Sakakibara, S. Takada, S.M. King, G.B. Witman, R. Kamiya, "A Chlamydomonas outer arm dynein mutant with a truncated beta heavy chain", J Cell Biol 122 (1993) 653-661.

[6] M.E. Porter, W.S. Sale, "The 9 + 2 axoneme anchors multiple inner arm dyneins and a network of kinases and phosphatases that control motility", J Cell Biol 151 (2000) F37-42.

[7] A. Gokhale, M. Wirschell, W.S. Sale, "Regulation of dynein-driven microtubule sliding by the axonemal protein kinase CK1 in Chlamydomonas flagella", J Cell Biol 186 (2009) 817-824.

[8] G. Habermacher, W.S. Sale, "Regulation of dynein-driven microtubule sliding by an axonemal kinase and phosphatase in Chlamydomonas flagella", Cell Motil Cytoskeleton 32 (1995) 106-109.

[9] G. Habermacher, W.S. Sale, "Regulation of flagellar dynein by an axonemal type-1 phosphatase in Chlamydomonas", J Cell Sci 109 (Pt 7) (1996) 1899-1907.

[10] G. Habermacher, W.S. Sale, "Regulation of flagellar dynein by phosphorylation of a 138-kD inner arm dynein intermediate chain", J Cell Biol 136 (1997) 167-176.

[11] D.R. Howard, G. Habermacher, D.B. Glass, E.F. Smith, W.S. Sale, "Regulation of Chlamydomonas flagellar dynein by an axonemal protein kinase", J Cell Biol 127 (1994) 1683-1692.

[12] P. Yang, W.S. Sale, "Casein kinase I is anchored on axonemal doublet microtubules and regulates flagellar dynein phosphorylation and activity", J Biol Chem 275 (2000) 18905-18912.

[13] E.F. Smith, "Regulation of flagellar dynein by calcium and a role for an axonemal calmodulin and calmodulin-dependent kinase", Mol Biol Cell 13 (2002) 3303-3313.

[14] G.B. Witman, "Isolation of Chlamydomonas flagella and flagellar axonemes", Methods Enzymol 134 (1986) 280-290.

[15] T.W. Hendrickson, C.A. Perrone, P. Griffin, K. Wuichet, J. Mueller, P. Yang, M.E. Porter, W.S. Sale, "IC138 is a WD-repeat dynein intermediate chain required for light chain assembly and regulation of flagellar bending", Mol Biol Cell 15 (2004) 5431-5442.

[16] R. Bower, K. VanderWaal, E. O'Toole, L. Fox, C. Perrone, J. Mueller, M. Wirschell, R. Kamiya, W.S. Sale, M.E. Porter, "IC138 defines a subdomain at the base of the I1 dynein that regulates microtubule sliding and flagellar motility", Mol Biol Cell 20 (2009) 3055-3063.

[17] K.E. VanderWaal, R. Yamamoto, K. Wakabayashi, L. Fox, R. Kamiya, S.K. Dutcher, P.V. Bayly, W.S. Sale, M.E. Porter, "Bop5 Mutations reveal new roles for the IC138 phosphoprotein in the regulation of flagellar motility and asymmetric waveforms", Mol Biol Cell 22 (2011) 2862-2874.