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Flow Cytometry Analysis of Cell Cycle in Myosin II-Deficient Yeast

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Objective. **To determine whether cell cycle changes can be detected in myosin II-deficient cells using flow cytometry techniques.**

Background. **Although the primary role of myosin II (Myo1p) in the yeast *Saccharomyces cerevisiae* is in cytokinesis we have reported that this conventional myosin also appears to influence the regulation of cell wall metabolism as indicated by increases in the expression of chitin metabolizing enzymes in a null mutant of the *MYO1* gene. The expression of these enzymes is known to be regulated in the cell cycle suggesting that cell cycle changes may alter their expression.**

Methods. **Flow cytometry was employed to assess the nuclear DNA content of logarithmic yeast cell cultures as a means of determining changes in the cell cycle of Myo1p-deficient cells.**

Results. **Significant changes were observed in the Myo1p-deficient strain suggesting that these cells are arrested in G2/M-phase of the cell cycle.**

Conclusions. **Based on the results of this preliminary study, we propose a model in which the increased activity of chitin metabolizing enzymes may be explained by a mitotic arrest in these cells.** *Key Words: MYO1, Myosin II, Cell Cycle, Flow Cytometry, Chitin.*

Cytoskeletal myosins have been classified based on sequence similarities among their motor domains (1). The class II myosins have a predicted structural and functional similarity to muscle myosins, giving them their classification as conventional myosins. Western blot analysis of the class II myosin heavy chain of *Saccharomyces cerevisiae* (Myo1p), with a specific polyclonal antiserum (2) identifies a set of 2-3 closely migrating bands with a predicted molecular weight of approximately 214 kDa. Most conventional myosin heavy chains fall within this typical molecular weight range. This protein possesses the structural hallmarks of conventional myosins as deduced from the predicted amino acid sequence, such as a globular head domain that contains the classical ATP and actin binding sites of the actin-dependent ATPase (3). An alpha helical rod domain spanning approximately one half of the molecule forms the C-terminus of the protein. This region is needed to form a coiled-coil structure in the putative heavy chain dimer. The assembly process for these putative Myo1p dimers and their association as bipolar filament structures is predicted to be driven by hydrophobic and electrostatic charge interactions between amino acids of this alpha helical coiled-coil domain. Although the capacity of class II myosin from *Dictyostelium discoideum* to associate into thick filament structures has been demonstrated *in vitro* (4), myosin thick filament formation has not been clearly demonstrated for Myo1p. The yeast Myo1p contains 7 helix-breaking proline residues within its rod segment which may alter the ability of this myosin to associate into typical myosin thick filaments. A similar domain of multiple helix-breaking prolines has been identified in the *Schizosaccharomyces pombe* class II myosin heavy chain, Myo2p (5). How these prolines affect filament assembly in yeast myosins is still unclear. However, the absence of Myo1p filaments does not seem to perturb the integrity of the actin cytoskeleton (our

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unpublished observations). Thus, the formation of actomyosin complexes does not appear to be essential for the stabilization of the actin filaments *in vivo*.

Studies conducted in other eukaryotes have described multiple functions for class II myosins such as cytokinesis (6,7), cell motility (8,9) and protection against osmotic stress (10). As in other eukaryotes, non-muscle myosins of various types have been identified in yeast (11). Myo1p is an important cytoskeletal component needed for cytokinesis during yeast cell division (12,13,14,15), resistance to osmotic stress, normal cellular morphology, and normal cell wall chitin distribution. Recent studies to identify the timing and localization of Myo1p in yeast shows that an actomyosin contractile ring forms at the neck between mother and daughter yeast cells early in the cell cycle and persists at this site until the end of mitosis following cytokinesis (15).

Myo1p-deficient yeast cells present delocalization of cell wall chitin, abnormal septa, and formation of attached cells which suggest that Myo1p may also be required in certain aspects of regulation of cell wall metabolism (13). Specifically, we have observed that Myo1p also appears to influence the regulation of chitin metabolism as indicated by increases in the expression of chitin metabolizing enzymes in a null mutant of MYO1 (16). Chitin metabolism is an essential part of the yeast cell division process. For example, degradation of the chitin plate (which forms the septum) that links mother and daughter cells is important for cell separation after cytokinesis. Chitinase 1 (Cts1p) is required in this process since a deficiency for this gene product causes the formation of attached cells (17,18). We have shown that Myo1p-deficient cells secrete much higher amounts of chitinase 1 than normal cells and that chitin synthesis is also elevated 2-fold in these cells. We have therefore proposed that the normal regulation of chitin metabolism is altered in Myo1p-deficient cells (16). As the enzymes involved in the synthesis of cell wall chitin have been shown to have various levels of regulation during the cell cycle (19), we have designed an experimental approach which explores the effects of Myo1p-deficiency on mitosis as the possible underlying cause of the deregulation of chitin metabolism in such cells.

Materials and Methods

The following yeast strains with their corresponding genotypes were used in this study: Haploid strain DBY745 : *MATα leu2-3, 112 ura3-52 ade1-101;* haploid strain BN4 : *MATα leu2-3, 112 ura3-52 ade1-101 myo1Δ:URA3* (3); and diploid strain PRY490 : *MATa /α his3-Δ200 /his3-Δ200 leu2-3,112 / leu2-3,112 trp1-Δ1/ rp1-Δ1 ura3-52/ura3-52* (kindly provided by Phil Robbins).

Strain BN4 was maintained at 30°C on Ura$^-$ drop-out broth media as described by Sherman et al. (20). YM-1 broth medium (1% yeast extract, 1% Bacto Peptone, 2% glucose, 1% succinic acid, 0.6% NaOH) was employed for the haploid and diploid wild-type strains DBY745 and PRY490 respectively.

To analyze the mitotic cycle of yeast cells, wild-type and Myo1p-deficient cell cultures were taken at similar cell densities (10^7 cells/ml) and a qualitative assessment was made of DNA contents by flow cytometry. Flow cytometric analysis of yeast cells was conducted by treatment of an aqueous suspension of cells, previously fixed in 70% ethanol at 4°C overnight, with 0.625 mg/ml Lyticase (Sigma) for 5-6 hours at 37°C to degrade cell walls and separate attached cells. In both the wild-type and Myo1p-deficient strains, greater than 90% efficiency of cell separation was achieved by this method. Subsequently, cells were suspended in a Phosphate Buffered Saline (pH 7.4), 1.2 M Sorbitol solution, treated with 2 mg/ml RNAse A for 1 hour at 37°C and with pepsin 10 mg/ml for 30 minutes at 37°C. Staining of 1 X 10^6 cells was done with a 0.05 mg/ml propidium iodide solution for 1 hour at room temperature followed by several washes with a Phosphate Buffered Saline (pH 7.4), 1.2 M Sorbitol solution to remove excess stain. Quantification of relative nuclear DNA content was performed by flow cytometric analysis of the stained cell preparations with UV fluorescence (488nm) on a Becton Dickinson FACSort as described by Haase and Lew (21). Wild type and Myo1p-deficient cell preparations were also examined by light microscopy and fluorescence microscopy (Nikon Labophot, at 480 nm) to quantify the contribution of any remaining attached cells to the overall percentage of cells scored in the G2/M population by flow cytometry. In both strains, these were observed to contribute less than 10% of the overall cell number scored in G2/M. This value includes 1-2% binucleated cells occasionally observed in the mutant cell cultures. Results of our flow cytometry analysis are expressed as relative fluorescence emitted (abscissa) versus total number of cells counted (ordinate).

Results and Discussion

In the flow cytometry analysis of a wild-type haploid cell population (Figure 1A), we observed that approximately one half of the cells contained a haploid DNA complement (N) indicating the proportion of wild-type cells in G1-phase of the cell cycle. The remainder of the wild-type cell population contained a two-fold

higher level of propidium iodide-induced fluorescence representing cells in G2/M-phase with a fully replicated DNA complement (2N). Upon our preliminary analysis of nuclear DNA content of Myo1p-deficient cells, these appear to have undergone M-phase arrest (Figure 1B). In this strain, the vast majority of cells in the population are dramatically shifted to the G2/M-phase (2N) with very few cells detectable in the G1-phase (N). Significantly, a substantial number of cells appear at a position of the histogram predicted to be (4N?). The flow cytometric profiles of the nuclei from the Myo1p-deficient cells comigrate precisely with the 2N and 4N positions corresponding to nuclear DNA of a diploid yeast strain (Figure 1C), thus supporting our interpretation that these represent nuclei with 2N and 4N DNA complement. Also, we have definitively excluded the possibility that an artefactual appearance of increased ploidy has been caused by attached-cell remnants generated through incomplete Lyticase digestion (data not shown). The obvious absence of G1-phase haploid nuclei from the Myo1p-deficient strain suggests that these cells are arrested in M-phase. In addition, the presence of a subset of cells with greater than 2N fluorescence suggests that there is an alteration in ploidy of Myo1p-deficient cells and furthermore, that myosin II-deficiency may has secondary consequences in the control of M-phase progression.

The metabolism of cell wall chitin has been shown to be regulated in a cell cycle-dependent manner (22,19). The best example where this regulation is most evident during mitosis is that of chitin synthase 2 and 3. Chitin synthase 2 levels peak at mitosis while the chitin synthase 3 localizes in cells (incipient and small budded cells) at early G1-phase, disappears in late G1-phase (large budded cells) and reappears once again as a ring at the neck between mother and daughter cells undergoing cytokinesis (23,24). On the other hand, the synthesis of CTS1 mRNA peaks at G1-phase (22) while the secreted chitinase 1 enzyme is essentially required for cell separation immediately following mitosis. Based on the results presented here, we propose that an M-phase arrest is caused by the inability of Myo1p-deficient cells to form an actomyosin ring and thus carry out normal cytokinesis. Furthermore, we infer that such an alteration in the timing of events during mitosis is also likely to alter the timing and level of expression of these chitin metabolizing enzymes in Myo1p-deficient cells. We have proposed a working model to accommodate this mutual relationship in which we implicate chitin synthase 2 and chitinase 1 as the primary effectors of these alterations (Figure 2). This model will be tested by the quantification of the activity of chitin metabolic enzymes and their respective mRNA expression levels under various conditions of induced mitotic arrest.

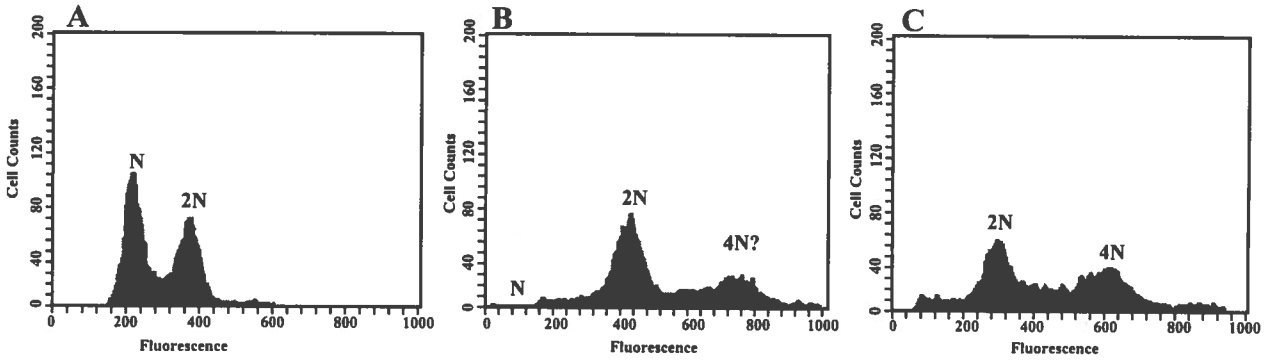

Figure 1. Flow cytometry analysis of DNA content in wild-type haploid (A), Myo1p-deficient haploid (B), and wild-type diploid (C) yeast cells.

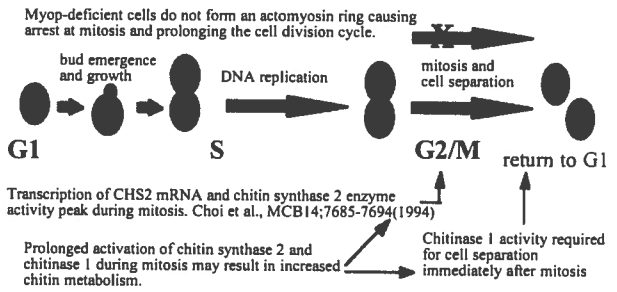

Figure 2. A model describing the progression of the cell cycle in normal yeast cells and a proposed interrelation between M-phase arrest and alterations in chitin metabolism in Myo1p-deficient cells.

Resumen

En este estudio nos propusimos determinar si existen cambios en el ciclo celular de levaduras deficientes en miosina tipo II (Myo1p) que fueran detectables mediante

la técnica de citometría de flujo. Nuestro interés surge de nuestros resultados anteriores donde observamos que la expresión de las enzimas que metabolizan quitina es alterada en una cepa mutante deficiente en Myo1p. Es sabido que la expresión de estas enzimas es regulada a través del ciclo celular. Se utilizó citometría de flujo como método para determinar el contenido de ADN nuclear en cultivos logarítmicos de células normales y deficientes en Myo1p lo cual refleja las fracciones relativas de cada población en la fase G1 versus G2/M del ciclo celular. Observamos cambios en la población deficiente en Myo1p indicando que estas células están arrestadas en la fase G2/M del ciclo celular. Basado en estas conclusiones preliminares, proponemos un modelo para explicar que el aumento en el metabolismo de quitina en células deficientes en la miosina II puede ser causado por cambios temporales en el ciclo mitótico.

References

1. Cheney RE, Riley MA, Mooseker MS. Phylogenetic analysis of the myosin superfamily. Cell Motil and Cytoskel 1993;24:215-223.
2. Segarra F M. Molecular and cellular analysis of myosin II expression in *Saccharomyces cerevisiae*. M.S. Thesis, Department of Biochemistry, University of Puerto Rico, Medical Sciences Campus, San Juan, P.R..1994.
3. Negron JA, Gonzalez S, Rodriguez-Medina JR. In vivo phosphorylation of class II myosin in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 1996;221:515-520.
4. Egelhoff TT, Randal LJ, Spudich JA. *Dictyostelium* myosin heavy chain phosphorylation sites regulate myosin filament assembly and localization *in vivo*. Cell 1993;75:363-371.
5. May KM, Watts F, Jones N, Hyams JS. Type II myosin involved in cytokinesis in the fission yeast, *Schizosaccharomyces pombe*. Cell Motil Cytoskel 1997;38:385-396.
6. DeLozanne A, Spudich JA. Disruption of *Dictyostelium* myosin heavy chain gene by homologous recombination. Science 1987;236:1086-1091.
7. Mabuchi I, Okuno M. The effect of myosin antibodies on the division of starfish blastomeres. J Cell Biol 1977;74:251-263.
8. Knecht DA, Loomis WF. Developmental consequences of the lack of myosin heavy chain in *Dictyostelium discoideum*. Dev Biol 1988;128:178-184.
9. Wessels D, Soll DR, Knecht DA, Loomis WF, DeLozanne A, Spudich JA. Cell motility and chemotaxis in *Dictyostelium* amebae lacking myosin heavy chain. Dev Biol 1988;128:164-177.
10. Kuwayama H, Ecke M, Gerisch G, VanHaastert PJM. Protection against osmotic stress by cGMP-mediated myosin phosphorylation. Science 1996;271: 207-209.
11. Brown SS.Myosins in yeast. Curr Opin Cell Biol 1997;9: 44-48.
12. Watts FZ, Shiels G, Orr E. The yeast *MYO1* gene encoding a myosin-like protein required for cell division. EMBO J 1987;6:3499-3505.
13. Rodriguez JR, Paterson BM. Yeast myosin heavy chain mutant: Maintenance of the cell class specific budding pattern and the normal deposition of chitin and cell wall components requires an intact myosin heavy chain gene. Cell Motil Cytoskel 1990;17:301-308.
14. Lippincott J, Li R. Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. J Cell Biol 1998;140: 355-366.
15. Bi E, Maddox P, Lew DJ, Salmon ED, MacMillan J, Yeh E, Pringle JR. Roles of a nonessential actomyosin contractile ring and of the septins in *Saccharomyces cerevisiae*. J Cell Biol 1998; in press.
16. Rodriguez-Medina JR, Cruz JA, Robbins PW, Bi E, Pringle JR. Elevated expression of chitinase 1 and chitin synthesis in myosin II-deficient *Saccharomyces cerevisiae*. Cell Mol Biol 1998;44:919-925.
17. Kuranda MJ, Robbins PW. Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. J Biol Chem 1991;266: 19758-19767.
18. Cabib E, Silverman SJ, Shaw JA. Chitinase and chitin synthase 1: counter-balancing activities in cell separation of *Saccharomyces cerevisiae*. J Gen Microbiol 1992;138: 97-102.
19. Choi WJ, Santos B, Durán A, Cabib E. Are yeast chitin synthases regulated at the transcriptional or posttranscriptional level ? Mol Cell Biol 1994;14:7685-7694.
20. Sherman F, Fink GR, Hicks JB. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1986.
21. Haase SB, Lew DJ. Flow cytometric analysis of DNA content in budding yeast. Methods Enzymol 1997;283:322-332.
22. Dohrmann PR, Butler G, Tamai K, Dorland S, Greene JR, Thiele DJ, Stillman DJ. Parallel pathways of gene regulation: homologous regulators SWI5 ACE2 differentially control transcription of HO and chitinase. Genes Dev 1992;6:93-104.
23. Santos B, Snyder M. Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myo2p. J Cell Biol 1997;136:95-110.
24 Chuang JS, Schekman RW. Differential trafficking and timed localization of two Chitin Synthase proteins, Chs2p and Chs3p. J Cell Biol 1996;135:597-610.