Mini-Review

Regulation of *Dictyostelium* Myosin II by Phosphorylation: What Is Essential and What Is Important?

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MUCH can be inferred about the function of a protein in vivo from its biochemical properties and its intracellular localization. The acid test for function in vivo, however, is the creation of mutant cell lines in which the protein of interest is either missing or nonfunctional and the analysis of the behavioral abnormalities exhibited by these mutant cells. One of the most dramatic successes in the use of this approach for the study of cell motility was the demonstration in 1987 that *Dictyostelium* cells which lack the heavy chain polypeptide for the conventional form of nonmuscle myosin, myosin II, have profound blocks in cytokinesis when grown in suspension and in multicellular development (6, 12). This elegant work was greatly aided by the simple fact that in *Dictyostelium* there is a single gene for the heavy chain of conventional myosin, so ablation of this gene or suppression of its expression with antisense RNA renders a cell that is missing an entire class of nonmuscle myosin. Subsequent work by a number of labs has shown that myosin II$^-$ cells are also unable to cap surface receptors crosslinked with Con A (22), have greatly reduced cortical stiffness (22), and are inefficient in cell locomotion and chemotaxis (37) (for review see reference 28). The fact that myosin II$^-$ cells exhibit these striking phenotypes, that wild type and mutated copies of the myosin II gene can be introduced into myosin II$^-$ cells, and that rescue of the behavioral abnormalities can be readily assayed, has opened the door to in vivo structure/function analyses of the myosin II heavy chain (7, 26, 35).

Dictyostelium myosin II is phosphorylated in vivo on both its heavy and light chains (1, 2, 15), and in vitro data indicate that these phosphorylations regulate the actin-activated ATPase, self-assembly properties, and motility properties of the molecule (for review see references 13 and 31). In the last several years, therefore, efforts by several labs have been directed at ascertaining the relative importance of these phosphorylations in vivo. In this short review, I will summarize the current in vitro data regarding the effects of heavy and light chain phosphorylation on *Dictyostelium* myosin II, and recent efforts to define the significance of these phosphorylations in living cells.

For heavy chain phosphorylation, a myosin II heavy chain kinase expressed in vegetative cells phosphorylates three threonine residues (residues 1823, 1833, and 2029) within the carboxyl-terminal portion of the myosin II α helical coiled-coil tail, which is the portion of the molecule that mediates self-assembly of myosin II monomers into small bipolar filaments (4, 18, 36). Myosin II molecules that are fully phosphorylated at all three sites are profoundly impaired in their ability to self-assemble into bipolar filaments at all ionic strengths (5, 15, 16, 23). A second heavy chain kinase, which is expressed only in developing cells, and whose sites of phosphorylation are unknown, has a similar effect on assembly properties (23). Heavy chain phosphorylation has also been reported to inhibit actin-activated ATPase activity, but this is almost certainly a consequence of the inhibition of filament formation (4, 32). Heavy chain phosphorylation may block filament formation by stabilizing a bent, assembly-incompetent form of myosin II (21, 31).

Egelhoff and colleagues (8) expressed in a myosin II$^-$ background myosin II heavy chains in which the three threonine residues were changed to either nonphosphorylatable alanine residues ("3XALA" myosin), or to aspartate residues ("3XASP" myosin). In the latter case, it was hoped that the negative charge on the aspartate residue would mimic phosphorylated threonine, thereby generating in a constitutive way "fully phosphorylated" myosin II. As anticipated, the 3XASP myosin was essentially incapable of forming filaments in vitro, while the 3XALA myosin assembled as well as wild type myosin "as isolated", or perhaps slightly better. These in vitro differences were mirrored in vivo, where 3XALA myosin was shown by analysis of triton-insoluble cytoskeletons to be dramatically over assembled relative to wild type myosin, while 3XASP myosin was under assembled. These differences in assembly state were also reflected in the extent and duration of myosin II localization at Con A caps. The most dramatic difference between these two engineered myosins, however, was that while 3XALA myosin was able to rescue the profound defects in cytokinesis, development and receptor capping found in myosin II null cells, the 3XASP myosin was not. One important conclusion from this work, therefore, is that the assembly of myosin II into bipolar filaments is a prerequisite for myosin II function in vivo. This conclusion was presaged by the early work of Delozanne and Spudich (6), who created *Dictyostelium* cells that make the heavy meromyosin (HMM)[1] fragment of my-

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1. *Abbreviations used in this paper*: HMM, heavy mermyosin; MLCK, myosin light chain kinase; RLC, regulatory light chain.

osin II instead of full-length myosin II. While HMM can support movement in vitro when artificially anchored to a surface, it cannot assemble into filaments (31). Consistent with this, HMM cells are indistinguishable phenotypically from heavy chain null cells (6, 19).

A second important conclusion from Egelhoff's work is that the ability to place phosphates on the three threonine residues in the tail is not essential for myosin II function. This does not mean that constitutively dephosphorylated myosin II works as well in vivo as myosin II which can be regulated by heavy chain phosphorylation. Indeed, some abnormalities in growth rate were reported for 3XALA myosin (most likely due to the over-assembly of this myosin) (8). Other subtle abnormalities would almost certainly be detected by further detailed analysis of these cells, since spatial and temporal changes in the assembly state of myosin II on a rapid time scale (i.e., seconds) are likely to be important for optimal execution of myosin II–dependent processes. It means simply that *Dictyostelium* cells can perform several functions that are absolutely dependent on myosin II when they express a myosin II that cannot be phosphorylated on its heavy chain.

Now, in two papers appearing in this issue (3, 20), Rex Chisholm's laboratory has explored the significance of the regulatory light chain subunit for myosin II structure and function, and, even more interestingly, the relative importance of regulatory light chain (RLC) phosphorylation in vivo. In the first paper (3), mutant cells which do not express the ~18-kD RLC were created by targeted disruption of the single-copy RLC gene, and these cells were then shown to resemble in most ways the behavior of myosin II heavy chain null cells (the sole difference being that RLC cells can still cap Con A–crosslinked surface receptors). These results, together with the recent work of Uyeda and Spudich (35), shed considerable light on the role that the RLC plays in the structure of the neck domain and the ability of the *Dictyostelium* myosin II head to function as a mechanoenzyme (see references 35 and 25). The results in the first paper also served to lay the ground work for the second paper (20), in which a comparison was made between the behavior of RLC$^-$ cells that were rescued with either wild type RLC or a nonphosphorylatable RLC. But before we get into this interesting story, a review of the in vitro data regarding the role of RLC phosphorylation in regulating *Dictyostelium* myosin II is in order.

In an early paper by Griffith et al. (10), evidence was presented using a partially purified *Dictyostelium* myosin light chain kinase (MLCK) that phosphorylation of the myosin II RLC regulates both the actin-activated ATPase and mechanochemical properties of myosin II. With regard to the actin-activated ATPase, myosin II whose RLC was completely dephosphorylated (<0.1 mole phosphate/mole RLC) following treatment with a protein phosphatase could be activated approximately sixfold (from ~20 nmoles/min/mg to ~120 nmoles/min/mg at high actin concentrations) by phosphorylating the myosin to ~1 mole phosphate/mole RLC (note that these and other ATPase numbers below are steady state values, not single turnover numbers, so the fold activation could be under estimated). Myosin II "as isolated", which typically has ~0.3 moles phosphate/mole RLC, had an intermediate specific activity (~60 nmoles/min/mg). This degree of regulation has been largely reproduced by Ruppel et al. (26) and Uyeda and Spudich (35) using the same MLCK, except that the kinase has now been expressed in *Escherichia coli* and purified to homogeneity (29, 30). Interestingly, Uyeda and Spudich (35) also showed that myosin in which the 30-residue portion of the heavy chain that contains the RLC binding site had been deleted, has an ~2.5-fold higher actin-activated ATPase activity than maximally activated wild type myosin. This result suggests that the complex of the RLC and its binding site within the "neck" domain of the myosin head serves to inhibit ATPase activity and that phosphorylation of the RLC partially derepresses this inhibition. In summary, then, it appears clear that phosphorylation of the *Dictyostelium* myosin II RLC has a significant effect on actin-activated ATPase activity. It is also clear, however, that unphosphorylated myosin II has a "reasonable" steady state actin-activated ATPase activity (~15–20% of the fully phosphorylated protein), and that the approximately sixfold activation of *Dictyostelium* myosin II by RLC phosphorylation is at least one order of magnitude less than the fold activation of smooth muscle and vertebrate nonmuscle myosins II by Ca^{2+}/calmodulin-dependent MLCK (using steady state ATPase numbers) (for review see references 27 and 31). Furthermore, while phosphorylation of the RLC of smooth muscle and vertebrate nonmuscle myosins converts folded, assembly-incompetent myosin (6S myosin) into unfolded, assembly-competent myosin (10S myosin) (34), phosphorylation of the *Dictyostelium* myosin II RLC does not appear to influence the assembly properties of the protein (10, 31).

With regard to the effect of RLC phosphorylation on motility, Griffith et al. (10) assayed for mechanochemical activity using myosin II–coated beads and the Nitella-based motility assay. Using their partially purified MLCK, they showed that beads coated with myosin "as isolated" moved at ~1 μm/sec, while beads coated with fully phosphorylated myosin moved at ~1.4 μm/s. This degree of activation of myosin "as isolated" by MLCK (~1.5-fold) has subsequently been reproduced with the cloned, purified MLCK, and the Kron/Spudich-type (14) motility assay (35). Of much greater significance is the fact that when Griffith et al. (10) coated beads with completely dephosphorylated myosin, they saw essentially no movement of the beads. This important result, which implies that RLC phosphorylation is absolutely required for myosin II to exhibit mechanochemical activity, has unfortunately not been repeated in the literature (the study by Uyeda and Spudich mentioned above [35] was done with partially phosphorylated myosin and fully phosphorylated myosin, but not with dephosphorylated myosin). The current situation would be remedied by a careful experiment in which completely dephosphorylated myosin II is phosphorylated with pure MLCK to various stoichiometries (ranging from 0–1 mole phosphate/mole RLC), and the speed with which these various samples move actin measured in the Kron/Spudich motility assay. As discussed in more detail below, such an experiment would shed additional light on the meaning of the RLC rescue experiments described by Ostrow et al. (20) in this issue.

So it is against this back drop of in vitro data that Ostrow et al. (20) set out on their RLC rescue experiments. After first establishing that wild type RLCs would rescue the severe phenotype of RLC$^-$ cells, they then set out to determine if successful rescue requires that the RLC be phos-

phorylatable. To do this they obviously needed to know the amino acid(s) within the RLC whose phosphorylation was responsible for the regulation of ATPase and mechanochemical activity. Unfortunately, no one has as yet directly determined which site(s) is phosphorylated in *Dictyostelium* myosin II RLCs that have been phosphorylated in vivo. Nevertheless, Ostrow et al. (20) have provided a convincing argument that serine 13 in the RLC is the major site phosphorylated in vivo in nonsynchronized, growing cells. First, they showed by NH_2-terminal protein sequencing that RLCs, which were made in *E. coli* and phosphorylated in vitro with the cloned MLCK, are phosphorylated on serine 13. When serine 13 was then converted into an alanine residue by site-directed mutagenesis (creating "S13A" RLC), and these RLCs expressed in *E. coli*, they could no longer be phosphorylated by MCLK in vitro. Moreover, when intact myosin II containing the S13A RLC subunit was purified from *Dictyostelium* transformants, it was also not possible to phosphorylate the heavy chain–associated RLC in vitro. In a final and very important control experiment, *Dictyostelium* cells were labeled metabolically with ^{32}p and myosin II was rapidly immunoprecipitated from whole cell extracts with an anti–heavy chain antibody. Whereas the RLC of immunoprecipitated wild type myosin contained significant amounts of radioactive phosphate, the RLC of S13A was essentially devoid of radioactive phosphate. This latter result provided strong evidence that serine 13 is the principal target for MLCK(s) in vivo.

As anticipated, Ostrow et al. (20) found that purified S13A myosin exhibited a basal actin-activated ATPase activity (~20 nmole/min/mg), equivalent to the activity reported for myosin II whose RLC had been fully dephosphorylated using a protein phosphatase (16). The big surprise was that cells expressing S13A myosin were essentially normal! Ostrow et al. (20) showed specifically that S13A myosin was able to rescue the defects in cytokinesis, development and myosin II heavy chain localization seen in RLC null cells. It would appear, therefore, that placement of phosphate on the light chain, as well as on the heavy chain, is not essential for myosin II–dependent functions in *Dictyostelium*. In the *Dictyostelium* knockout business, the normal explanation given for relatively subtle phenotypes, which is the existence of multiple, functionally redundant isoforms (11), simply does not apply to the analysis of cells expressing non-phosphorylatable myosin II molecules. It would seem, therefore, that as long as *Dictyostelium* myosin II can assemble into filaments, it can support functions that are absolutely myosin II–dependent even when the filaments are not particularly active.

With regard to light chain phosphorylation specifically, how surprising are the results of Ostrow et al. (20)? If one considers only the effect of RLC phosphorylation on the actin-activated ATPase of myosin II, the results are perhaps not totally surprising, since the relatively small activation of steady state ATPase (approximately sixfold) is almost more modulatory than regulatory (especially compared to the RLC-based regulation of vertebrate smooth and nonmuscle myosins). It is likely, however, that the real degree of activation of *Dictyostelium* myosin II by RLC phosphorylation, which would be more accurately determined using single turnover experiments (33), is considerably higher than the approximately sixfold number obtained from steady state measurements. If one also considers the early results of Griffiths et al. (10), which suggest that RLC phosphorylation is required for myosin II to move on actin (i.e., that for dephosphorylated myosin II, ATPase, and motility are completely uncoupled), then the results of Ostrow et al. (20) become truly amazing. As mentioned above, however, that early finding has not been repeated, and for now should perhaps be viewed with some skepticism. Indeed, the best way to unequivocally answer this question would be to use the S13A myosin made by Ostrow et al. (20) in a motility assay!

While heavy and light chain phosphorylation of myosin II are not essential for *Dictyostelium* cells to perform several myosin II–dependent functions, it would be a mistake to conclude that these phosphorylations are not important for optimal cellular function. The modulation of myosin II assembly and ATPase activity by these phosphorylations could very easily improve the efficiency of many cellular processes to an extent which, while difficult to measure in a statistically significant way, would provide a striking selective advantage over time. For example, as pointed out by Ostrow et al. (20), a 5% increase in the efficiency of cytokinesis, which might well be the difference between regulated and nonregulated myosin II, would result in a growth advantage that would manifest itself on a time scale of weeks to months. Obviously, even minute improvements in the efficiency of processes that either require myosin II or simply involve myosin II, would on an evolutionary scale provide a strong selective advantage. Indeed the mere fact that light and heavy chain phosphorylations exist for *Dictyostelium* myosin II strongly suggests that they evolved because of the selective advantage that they provide.

The next logical step in the analysis of these myosin II phosphorylation site mutants is clearly the application of functional tests that can detect subtle differences between control and experiment cells. For example, detailed motility assays of cells expressing 3XALA myosin and S13A myosin using quantitative video microscopy could very well detect important differences in the speed and orientation of crawling cells and in the dynamics of their shape change (37). Such tests could also be performed under artificial "load" conditions (e.g., locomotion on a highly adhesive surface) which may amplify the differences between mutant and wild type cells. Application of functional tests which yield graded, quantifiable responses will also be useful in defining differences between wild type cells and phosphorylation site mutants. These could include measurements of cortical tension (22) and measurements of the speed with which cytoskeletal preparations can contract (17). It seems highly likely that the tendency for 3XALA myosin to over assemble in vivo, and the low ATPase activity exhibited by S13A myosin, will be reflected in some of these assays of whole cell behavior.

In addition to the studies described above, the ability to distinguish phosphorylated myosin II from dephosphorylated myosin II using antibodies that are specific for each form, when coupled with immunofluorescence localizations, would be of great benefit in understanding the role of myosin II phosphorylation in cell function. Just such an approach has recently been used by Baines and co-workers (Baines, I. C., A. Corigliano-Murphy, and E. D. Korn, manuscript submitted for publication) for several of the *Acanthamoeba* myosin I heavy chain isoforms. Secondly, while the immuno-

precipitation data of Ostrow et al. (20) provides strong evidence that serine 13 in the RLC is the principal site for phosphorylation in growing cells, this data does not absolutely rule out other physiologically important phosphorylations of the RLC (Smith, J. L., L. A. Silveira, and J. A. Spudich. 1992. *Mol. Biol. Cell.* 3:45a; Silveira, L. A., J. A. Smith, and J. A. Spudich. 1993. *Mol. Biol. Cell.* 3:44a). Some of these phosphorylations might only be detected in immunoprecipitated myosin when cells have been synchronized to divide or undergo chemotactic aggregation. Myosin II may also be compartmentalized in cells, so it is possible that a phosphorylated, active population of myosin II molecules that are critical for cell function could be largely missed in immunoprecipitation experiments if the bulk of the cellular myosin II is unphosphorylated. Furthermore, some of these additional, putative RLC phosphorylations may have their desired effect in vivo at relatively low stoichiometries. Finally, much work is needed to elucidate how the light and heavy chain kinases for *Dictyostelium* myosin II are regulated by signal transduction pathways. In this regard, the two myosin II heavy chain kinases sequenced so far reveal interesting motifs in their primary structure which probably relate to how they are regulated in vivo (9, 24).

The author thanks Graham Côté, Jim Sellers, Tom Egelhoff, Janet Smith, and Edward Korn for their comments on the manuscript.

Received for publication 4 November 1994 and in revised form 12 November 1994.

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